Selection for the bacterial capsule in the absence of biotic and abiotic aggressions depends on growth conditions

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ABSTRACT

Extracellular capsules are the outermost layer of some cells and are prevalent across Bacteria. They protect the cell against both abiotic and biotic stresses such as bacteriophages and the host immune system. Yet, it is unclear if capsules contribute to fitness in the absence of external aggressions, in spite of the cost of production. Here, we enquire if there are conditions favouring the presence of the capsule in *Klebsiella*, where it is a major virulence factor. We allowed independent populations of 19 different strains, representing six different capsule serotypes, to evolve for twenty generations in different growth media. We observed that capsules were maintained in nutrient-poor media but were selected against in nutrient-rich media. Competitions with capsule mutants in nine different strains showed that capsules provide *ca* 20% of fitness advantage in nutrient poor-conditions. This is not caused by differences in maximal growth rate, nor death rates. We showed that most capsulated populations reached higher yields. We further characterized how growth conditions affected the role of the capsule during pathogenensis and whether this is conserved across *Klebsiella* strains. Our data reveals that the environment and the serotype strongly influences major virulence traits such as hypermucoviscosity and biofilm formation. Our data shows that the capsule can be selected for in situations lacking abiotic, but also biotic stresses. This raises the possibility that the forces selecting for the presence of a capsule are different from the ones selecting for rapid serotype variation.

IMPORTANCE

Bacterial capsules are a wide-spread virulence factor that limits efficacy of antimicrobial therapy. Whereas most studies focus on the role of the capsule in pathogenesis, very few have addressed the conditions under which the capsule is primarily selected for. Here, we show that small changes in growth media have a strong impact in the maintenance of the capsule and the fitness advantage they confer. Our results suggest that the forces selecting for the capsule may be different from those selecting for its composition diversification, namely the immune system and phage predation. Our results further support that the role of the capsule in virulence may be a by-product of adaptation, hinting that there may be other functionalities yet to be discovered for it.

INTRODUCTION

Most environments are complex and can be temporally or spatially heterogeneous, both in their abiotic (pH, nutrients, chemicals…) or biotic compositions (niche invasions, extinction events). This can represent an evolutionary challenge for microbes (1, 2). In order to survive, bacterial species can either move to less stressful territories, adapt to the new circumstances or resist until conditions are suitable (3). Notably, bacteria can produce a thick extracellular layer – the capsule – that has been reported to enhance survival and tolerate external aggressions (4, 5). Extracellular capsules constitute the first barrier between the cell and its environment and are encoded in half of the bacterial genomes, in some archaea and yeast (6). Capsules fulfill many roles. They have been associated with resistance to abiotic stresses such as UV light and desiccation (4, 5). However, they are mostly studied for their role enhancing survival under biotic stresses, including resistance to grazing protozoa (7), antibiotics (8, 9), antimicrobial peptides (10, 11), and host immune defences, such as human serum and phagocytes (12-14).

The majority of capsules belong to Group I or Wzx/Wzy-dependent capsules (6). These capsules are high molecular weight polysaccharides made up of different repeat units of oligosaccharides. The different possible oligosaccharide combinations and residue modifications lead to different serotypes. Serotype diversity, even within-species, is very

large. This genetic diversity has been particularly studied in facultative pathogens such as *Streptococcus pneumoniae* (15), *Campylobacter jejuni* (16), and *Acinetobacter baumanii* (17). In *K. pneumoniae,* there are at least 77 serologically-defined capsule serotypes (18-20), and more than 130 serotypes identified through comparative genomics (21, 22). This serotype diversity results from the faster evolutionary rates of capsular loci compared to those of the rest of the genome. The latter has been shown to occur by homologous recombination (23, 24) and horizontal gene transfer (25-27). As a result, the same capsular serotype can be found in multiple lineages of *K. pneumoniae* and a monophyletic clade can include distinct serotypes (24, 28). Selection for rapid variation of capsule serotypes is thought to result from biotic stresses, notably because phages and the human adaptive immune system target the capsule in a serotype specific manner, which favours the emergence of novel serotypes (29). This process can be accelerated by vaccines which target specific capsule serotypes (16, 30, 31).

Many studies have assessed the role of the capsule during pathogenesis, yet little is known about the primary selective forces that underlie the emergence and maintenance of the capsule when bacteria are not under biotic stress. Since capsules are costly to produce, if they are selected to protect bacterial cells from phages, immune systems, or specific stresses, they should be readily lost in the absence of these challenges. Consequently, serial passaging of *Klebsiella* cultures in nutrient-rich laboratory media (LB), as well as aging of cultures, readily leads to the emergence of non-capsulated clones (32, 33), indicating that the capsule can be easily lost.

To further investigate the conditions where the capsule is advantageous, we study bacteria from the genus Klebsiella. This genus is composed of ubiquitous bacteria able to colonize a large range of environments (34), due to a diverse metabolism (35) and the presence of a Wzx/Wzy capsule (21). To date, most, if not all isolated *Klebsiella*, have a capsule operon. *Klebsiella spp*., and more particularly *K. pneumoniae*, are opportunistic pathogens and major multi-drug resistant (MDR) bacteria that cause both community-acquired and hospital-acquired infections in humans (36-38). The capsule is regarded as the major virulence factor of the genus (39). To test the maintenance of capsule production in several growth conditions, including host-related environments, we used nineteen *Klebsiella* strains representative of the species genetic diversity (35). These strains have different capsule serotypes (K-locus) and O-antigens (O-locus), and include a closely-related *K. variicola* (strain #24), and two *K. quasipneumoniae* subsp. *similpneumoniae* (strains #44 & #214) (Table S1). We then

generated a panel of nine capsule mutants to address the precise fitness effects of the capsule in a range of conditions. Finally, we explored the importance of the serotypes and the genetic background of the strain in driving capsule-related phenotypes. The integration of these results revealed that the capsule can be selected in the absence of biotic stresses.

RESULTS

The capsule is lost in nutrient rich environments

Early reports (32, 33) and our laboratory observations reveal that serial passaging of *Klebsiella* cultures in rich laboratory media (LB) generates spontaneous capsule mutants (40). This strongly suggests that capsule has a negative impact on fitness in laboratory environments. To understand the fitness cost of capsule in the absence of biotic pressures, we serially passaged 19 different *Klebsiella* strains belonging to six different serotypes (at least three strains per serotype), amongst which the virulence-associated KL1, KL2 and the emergent KL107 associated to multi-drug resistance. We then measured the natural emergence of non-capsulated mutants after *ca*. 20 generations. We tested the emergence of non-capsulated mutants in five different growth media (see Methods). Artificial sputum medium (ASM) (41) and artificial urine medium (AUM) (42) mimic host-related nutritional conditions. The remaining are typical laboratory media with decreasing levels of nutrients: rich Luria-Bertani medium (LB), minimal medium supplemented with 0.2% of glucose (M02), and minimal medium supplemented with 0.05% of glucose (M005). Beforehand, we verified that the strains were able to produce a capsule in our growth conditions by direct microscopic observation and by capsule quantification using the uronic acid method (Methods, Figure S1). To initiate the experiment, we grew three independent clones of each strain for 16 hours in LB and then diluted (1:100) into each growth media. All 19 strains were then serially transferred into fresh media every 24 hours for three days. Although each growth media has different carrying capacities, cultures were always diluted to 1:100 and after 24h of growth, each media was saturated, ensuring that in all growth media, the different populations underwent the similar number of generations (~ 20).

After three days, in 111 of the 290 independently evolved populations (38%) non-capsulated mutants emerged (Figure 1). Most ASM (74%) and LB (89%) lineages showed capsule mutants, and these accounted for 85 of the 111 populations. In contrast, non-capsulated

mutants were very rarely observed in the other three growth media (M02, M005, and AUM. These data suggest that it was the amount of nutrients present in the growth media that drove the maintenance of capsule rather than the opposition between host-related environments versus lab environments. Indeed, it was in the nutrient rich media (ASM and LB), where the capsule was readily lost, whereas it was consistently maintained in the three nutrient poor media (M02, M005 and AUM). To further confirm this, we allowed the experiment to run for 30 days in LB and M02 for a KL2 strain (#26). The three replicate populations that evolved in M02 remained capsulated whereas populations evolving in LB rapidly lost their capsule and remained so until the end (Figure S2). Some strains also seem more prone to lose the capsule. An extreme example concerns strain #62 for which we found capsule mutants in all fifteen independently-evolved populations across the five different growth media. We tested if these differences could be due to the amount of capsule produced by each strain, but the frequency of non-capsulated mutants does not correlate with the amount of capsule produced by each strain ($P > 0.05$, Spearman's rank correlation). We tested the relevance of both serotype and growth media on the emergence of capsule mutants using a generalized linear model ($R^2 = 0.39$). This revealed that the emergence of capsule mutants was both dependent on the growth media ($P < 0.001$) and on the serotype ($P<0.02$). This suggests the capsule is deleterious under rich media and selected for in poor media, a factor that depends at a lesser extent on the capsule serotype.

The capsule provides a fitness advantage in nutrient poor environments.

To precisely assess the costs and benefits of the capsule across growth media, we sought to perform competition experiments between capsulated (Cap+) and their respective isogenic non-capsulated (Cap-) mutants. We thus undertook the construction of in-frame and markerless deletions of *wza*, the outer membrane exporter of the capsule, a well-annotated and essential core gene of the capsule operon. We used the allelic recombination strategy and aimed at deleting *wza* in 15 different strains from different serotypes, for which we had easily obtained *rcsB* in-frame deletion mutants. (RcsB is a capsule regulator and mutants have a reduced capsule production (43, 44). We obtained *wza* deletion mutants for five strains, from several serotypes (see Table S1). From these double-recombination events (see Methods), we isolated both a mutant and a wild type. This ensures that the phenotype observed was directly due to the *wza* deletion and not to other off-target SNPs that could have accumulated in the genome during the mutant generation, and thus present in both the mutant and the wild type.

All experiments were performed in parallel with the parental strain, *Δwza* mutant and the associated wild type from the same double-recombination event. After several attempts, we obtained *wza* deletion mutants in four other strains, but Illumina sequencing revealed off-target compensatory mutations only present in the mutant (and not in the associated wild type) (see Methods). Thus, because we cannot be certain that the phenotypes are exclusively due to the deletion of *wza*, we present general statistics in the text and supplemental figures, but only present results for the five clean mutants in the main figures (see Methods). Microscopic observation and uronic acid quantification confirmed that all nine *Δwza* mutants did not produce a capsule (Figure S3). For all other seven strains, no *Δwza* mutants were obtained despite several attempts.

To assess the competitive indexes, or fitness, of the mutant strains, we performed direct competition between wild type strains and their respective the *Δwza* mutant strains. They were initially mixed in a 1:1 ratio and allowed to compete in all five growth media for a 24-hour period. We estimated that the natural emergence of capsule mutants in rich media is rare during the first 24 hours and is thus negligible in the interpretation of our results (see Methods). Competition experiments showed a significant effect of the environment in the fitness of the Cap + strains (Figure 2, Kruskal-Wallis, $P = 0.0046$). In agreement with our previous results that showed the maintenance of capsule in nutrient poor media, Cap+ strains had a strong fitness advantage in M02, M05 and AUM ($P< 0.05$, $N=8$, one-sided t-test, difference of 1). In this analysis, we excluded strain #55 due to a growth deficiency of the *Δwza* mutant (see Methods). The average fitness advantage provided by the capsule in nutrient poor media is *ca.* 20%. Opposite to all other strains, both deletion mutants of KL24 strains showed increased fitness in AUM, suggesting a particular benefit of the capsule in these two strains in AUM (Figure S5). On the other hand, in nutrient rich media, we observe a significant decrease of Cap+ genotypes ($P= 0.07$ and $P = 0.05$ in LB and ASM, respectively, one-sided t-test, difference of 1). Decreased fitness in these growth media was significant in 5 out of 8 strains in LB and 4 out of 8 in ASM strains. More particularly, *K. variicola* is significantly less fit in nutrient rich media (-25%). Similarly, the KL1 strain (#56) is 18% less fit. Two other strains, KL2 and KL124, also have significant disadvantages in LB and ASM, respectively ($P<0.05$, one-sided t-test, difference of 1) (Figure 2 and Figure S5). Taken together, our results confirm that the capsule is advantageous in nutrient-poor media, but not in rich media.

The capsule increases the population yield in nutrient poor growth media

We investigated how the capsule could increase fitness in nutrient poor environments by assessing the effect of mutants in a number of conditions. We first tested whether the capsule provided an advantage during starvation, by increasing cell survival, as shown in *Streptococcus pneumoniae* (45). To check this, we followed the survival of capsule mutants and wild type under starvation conditions (see Methods). We allowed cells to starve for three months and counted surviving CFUs every month. As expected, mortality rates of *Klebsiella* are very low compared to other Enterobacteria (46). Yet, we observed no significant differences between wild type and the capsule mutants after 30 or 60 days (Figure 3A and Figure S5A). To further check a potential effect of the *Klebsiella* capsule on survival, we also challenged the strains to desiccation. No effect of the capsule on cellular survival was observed during desiccation ($P > 0.05$, t test for significant difference between mutants and wild type, Figure S5B).

Given that no significant effect of the capsule on cellular survival was observed, we tested whether differences in growth rate could account for the fitness effects observed. We thus allowed all strains and their mutants to grow in microtiter plates in all growth media and calculated the minimum generation time (Figure 3B and Figure S6A). As expected, we observed significant differences in growth rate across strains and environments (GLM, $P < 0.001$ for both, $R^2 = 0.60$). However, no significant differences between the mutants and capsulated strains was observed ($P = 0.41$). Thus, increased fitness in nutrient poor media s is not due to faster growth. Close inspection of the growth curves revealed that strains with capsule in nutrient poor media reached higher absorbances (ODs), a proxy for population yield (Figure 3C and Figure S6B). Control experiments showed a strong correlation between absorbance and cell numbers (see Methods). Furthermore, direct plating of cells after 16 hours of growth in microtiter plates also revealed larger population sizes in capsulated strains than in mutants. Direct comparison of yield between mutants and the respective wild type showed significant difference for several strains in nutrient poor environments. Taken together, there is a statistically significant difference in yield between the mutants and the wild type in nutrient poor media ($P < 0.01$ in M02, $P < 0.001$ in M005 and $P = 0.1$ in AUM, paired t-test, $N = 8$), suggesting that the fitness benefits observed in competition are due to an increased yield or reproductive lifespan.

To further understand how capsulated bacteria achieve higher yields in poor media, we first verified that in our experimental conditions, non-capsulated strains did not exhibit higher death rates (Two-way ANOVA, P = 0.47, Figure S6C). We then hypothesized that capsulated bacteria could use their capsule as a nutrient source. To do so, we constructed new capsule mutants (*ΔwcaJ* and *ΔwbaP*, initial glycosyl transferases*)*, to interrupt capsule production at the first biosynthesis step and avoid capsule build up that could interfere with our results. We allowed the mutant strains to grow for 48 hours in minimal media with no source of carbon, in the presence of purified capsule added exogenously and of uronic acid used as a standard to quantify capsule production (Figure S7). We did not observe significant differences between the treatments where the capsule was added exogenously and the respective controls (see Methods, see Figure S7). These results suggest that neither increased cellular death of mutants nor the ability to consume the capsule explain the observed higher yield.

Capsule mutants undergo different fates in host-mimicking media

Our previous results show that small changes in growth conditions can strongly affect selection for the capsule. We thus sought to further understand how these growth conditions affect the role of the capsule during pathogenesis and whether this is conserved across Klebsiella strains. We first addressed the ability of non-capsulated mutants to resist to human serum. Three Cap- mutants were significantly more sensitive to human serum than their Cap+ counterpart, whereas for the other two strains there was no significant difference (Figure 4A). A KL2 strain (#26) was resistant to human serum irrespective of its capsule (Figure 4A). One KL24 strain (#51) (Figure S8A) and *K. variicola* (strain #24) Cap- was as sensitive as the Cap+. The capsules of strain #211 and of strain #37, from the same serotype as strains #24 and #37, respectively, provide a significant degree of protection. These results strongly suggest that the effect of the capsule on resistance to serum is strain-dependent (Multifactorial ANOVA, P < 0.001).

We then explored the ability of capsule mutants to survive in the presence of primary (sodium cholate –CHO-) and secondary (sodium deoxycholate –DCH-) bile salts. At physiological concentrations (0.05%), we observed no significant sensitivity of *Klebsiella* to bile salts (Figure S8B). When treated with 10X physiological concentrations, some capsule mutants showed reduced viability, whereas others were not (Figure S8B).

Finally, the ability of niche colonization, specially within a host, is directly linked to the ability of the strains to adhere surfaces and proliferate, that is, to form a biofilm. Cells within biofilms are more resilient, tolerant to antibiotics and less exposed to the host immune system (47). We thus assessed the ability of the strains to form biofilm and how the capsule could impact this trait. It has been previously shown that in some strains of *Klebsiella*, capsule production masks the necessary fimbriae required to attach to the host mucosa (48, 49). On average, we do not observe a difference in the amount of biofilm that the capsulated and not capsulated mutants form ($P > 0.05$, $N = 9$, paired t-test, in all different growth media). However, we observe a strong negative correlation between the amount of biofilm the parental strain forms and the difference between the capsule mutant and its associated wild type (Figure 4B). Deletion of the capsule in strains with limited ability to form biofilm results in increased ability to form biofilm. In other words, the capsule tends to contribute more positively to biofilm formation in strains forming more biofilm. At the strain level, we observe that in some cases the non-capsulated mutants adhere significantly more across all environments (strain #56) (Figure S9), whereas other strains have a stronger ability to form biofilm when the capsule is present (strain #47) (Figure S9). This might either result from the absence of adhesion factors in the strain or it could also suggest that depending on the capsule composition, it could act as an adhesion factor. Overall, our data suggest that it is not the presence or absence of the capsule that affects the ability to form biofilm, but rather the amount of capsule expressed by a given strain.

Taken together, these results show that some properties like resistance to bile salts or human serum, are not inherent to the presence of the capsule, but are rather strain-dependent, or dependent on the serotype-genome interaction.

Complex interactions between the environment, genetic background and serotype

To further understand the interactions between the serotype, genetic background and environment, we used the original dataset of 19 strains, in which there are at least three strains belonging to each serotype. We analyzed different capsule-related variables in all five growth media. In addition to the amount of capsule production (Figure S2), the natural emergence of non-capsulated mutants (Figure 1), maximum growth rate, the maximum yield and biofilm

formation, we also measured the hypermucoviscosity of strains, a trait linked to hypervirulence and driven, at least partly by the amount of capsule (50) (Figures S10-S12). Firstly, we used Principal Components Analysis (PCA) to analyze how the different factors (media, serotype or strain) explain the observed variance in the data. The first and second principal components explained more than 60% of the variation observed (Figure 5A). We observed no clear grouping of serotypes according to the two first axes. This suggests that the serotype is not a major determinant of the capsule-related phenotypes (Figure S13). The ellipses regrouping the points by type of media show a small overlap between both rich media (ASM and LB) and these are clearly separated from the two poorest media (M005 and AUM). This suggests that most of the variance is explained by the media, and more presumably by the amount of nutrients available (Figure 5A). We then used multifactorial ANOVA to analyze quantitatively the effect of the environment and the serotype on each trait. As expected, we observed a significant effect of the environment in all measured traits, except in biofilm formation ($R^2 = 0.57$, $P = 0.06$, Table S2). As suggested by the PCA, the influence of the serotype in the capsule-associated traits is less important. Interestingly, the terms of interaction between the environment and the serotype are not statistically significant, suggesting that they are independent (Table S2).

Secondly, we sought to determine how the different capsule-related variables correlated with each other. The PCA analysis already suggested the existence of such correlations (Figure 5B). As expected, we observe a strong correlation between hypermucoviscosity (HMV) and the amount of capsule, in all individual growth media except in M005. Consistently with previous reports (51, 52), we observe that the population yield is inversely proportional to the growth rate (Figure 5B). This is significant in all growth media, except M005 (for which $P = 0.07$). Further, the amount of capsule is positively correlated with the maximum yield (Figure 5B), supporting our observation that the capsule contributes to increased yield of the population. In contrast, there is a negative correlation between the amount of capsule and the generation time. These results are driven by growth in nutrient poor environments (rho < -0.2 and rho > 0.2 in nutrient poor and nutrient rich environments) (Figure 5B). More precisely, in poor media, bacteria with more capsule are able to grow faster that those producing less capsule. This was not evident from the mutant analysis, as these showed no significant differences in generation time between the capsulated and non-capsulated strains. Overall, our results show that the capsule may impact several important cellular traits, like mucoviscosity and growth, but this is strongly dependent on the environment.

DISCUSSION

The capsule is an extracellular structure commonly associated to the ability to resist biotic or abiotic aggressions, like the immune system, phages, or desiccation (10, 39, 53). Here, we explore further the conditions in which the capsule can be selected for, despite the cost of its production. Our results show that simple abiotic factors, such as nutrient limitation, can change dramatically whether the capsule is maintained in a population. More precisely, the capsule can be selected for in the absence of biotic stresses or strong abiotic aggressions. During selection for fast growth in nutrient-poor environments, capsulated cells have strong fitness advantages (*ca*. 20%). These challenges the current view that the main role of the capsule is to withstand aggressions. Our previous work already suggested that its role in virulence may be a by-product of adaptation, raising the question of its primary function in cells (6). Here we further show that, even in the absence of strong aggressions, the capsule provides a notorious advantage and may be selected because of the advantage it provides during growth.

We hypothesized that the cost of the capsule could be reflected in a reduced growth rate between of wild type strains compared to their respective capsule mutants. We, however, do not observe such differences, as calculated by the minimum generation time (Figure 3B and 6B). One possible explanation could be the lack of sensitivity of absorbance tests or to the large variation across biological replicates (over 10 %). However, our results are in line with studies performed in other species, for instance, in *Acinetobacter baumanii*, where the growth of wild type and capsule mutants in laboratory media was similar (54). Experiments performed in *S. pneumoniae* showed differences in growth rate between wild type and non-capsulated mutants, but these were dependent on capsule serotype. The loss of capsule 7B increased significantly the growth rate in rich media whereas the opposite was true for capsule 6B (55). The lack of observable differences may be due to the low cost of the production of capsule, or to the fine-tuning of its complex regulatory network (56). Indeed, the capsule could be regarded as an essential constituent of the physiology of *Klebsiella spp.* as, so far, few unencapsulated strains have been isolated, and the genome could have minimized the cost of its production. Despite the absence of differences between capsulated and unencapsulated strains, significant differences are observed across strains and environments, most notably, we

observe a negative correlation between the amount of capsule and generation time in nutrient poor environments (Figure 5B). Thus, bacteria producing more capsule are able to grow faster in nutrient poor environments, again highlighting the central role of the capsule in such environments and suggesting that that there may be other unknown functions to the capsule.

The increased fitness in nutrient-poor environments can be explained by the differences in population yield, or the increase of the reproductive lifespan of the population, as determined both by CFU and absorbance. In nutrient-poor environments, capsulated cells achieve higher yields and are fitter. Reduced yield in capsulated mutants was already observed in strains of serotype 6B of *S. pneumoniae* (55). How capsulated bacteria achieve a higher yield is still not clear, but could be due to several reasons. We first hypothesized that the capsule could increase survival in our cultures. The advantage provided by the capsule could be associated with a growth advantage, or reduced death, in stationary phase (GASP phenotype), a period in which birth and death rates are balanced. However, our results do not support this (Figure S6C). The phase referred to as "death phase" during growing cultures typically sets in after two or three days of batch culture in other Enterobacteria such as *E. coli* (57), and we observe higher yield after only 16 hours. Further, our long-term starvation experiments revealed no differences in survival between capsulated and non-capsulated strains. Previous reports had already shown that in the long term, *Klebsiella spp* are very resilient and have very slow death rates (58, 59), including in lake water at temperatures close to 0 (60) or during long-term starvation in PBS as a member of an oral community (46). Second, capsulated bacteria could use the capsules' polysaccharides to further multiply after consumption of all the environmental resources, as previously reported in *S. pneumoniae* (45). Addition of exogenous capsule to non-capsulated mutants did not result in a significant increase of CFU in a population, suggesting that it does not allow for higher population yields. Third, our analysis shows that biofilm formation, capsule amount and hypermucovicosity are positively correlated with yield (Figure 5B). This suggests that capsules could promote aggregation and thus cells could be present at higher local densities, due to limited dispersion and clumping during growth, which would in term, boost their individual fitness and increase total population yield. For example, dead capsulated cells would be dispersed less and could be more readily consumed by neighbouring growing cells.

Further analysis of our panel of strains reveals that capsule-associated features related to virulence are very variable across all strains and serotypes. First, we observe an overall strong

positive correlation between the capsule and hypermucoviscosity (HMV), a proxy of hypervirulence. This supports the hypothesis that HMV is a result of capsule overproduction. Yet, a recent study has shown that capsule overproduction is not required for HMV (61), suggesting that the role of the capsule in HMV is convoluted or dependent on the strain (56). Indeed, we observe an effect of serotype in HMV (P = 0.002). Second, our results are in agreement with a previous study (62) in which cells from different serotypes were first exposed to depolymerases, to digest the capsule, and then tested for resistance to human serum and phagocytosis. Consistently, some non-capsulated strains were more sensitive to human serum, but others seem to be as resistant as the control cultures to which no depolymerase was previously added. Resistance to human serum can also be due to the presence different O-antigens (63, 64). In our panel, only two strains #211 and #26 share the same O-antigen (O1v1). These strains have different resistance profiles, suggesting that O-antigen is not solely responsible for serum resistance. Third, we do not observe any protection of the capsule from bile salts at physiological levels, as *Klebsiella* strains here tested seem to be naturally resistant to bile salts. When bile salt concentrations are increased by a factor of ten, the capsule significantly increases survival in some cells but not all. Overall, these results highlight that the capsules do not have the same function in all *Klebsiella* strains, and are thus, non-universal. Further, it highlights that the absence of capsule may not necessarily affect the potential of these strains for pathogenesis. A recent study showed that compared to capsulated carbapenem-resistant *K. pneumoniae* ST258 isolates, the unencapsulated isolates formed more biofilm and could invade the epithelial bladder tissue more effectively. This resulted in the formation of intracellular infective reservoirs that lead to recurrent urinary infections (65).

To conclude, we show that the growth media affects the relative fitness of capsulated bacteria, which may justify selection for capsulated bacteria in nutritionally poor environments. One cannot ignore the possibility that such a selection pressure may drive capsule diversification, e.g. different capsules could be better adapted to certain poor environments because of their different propensity to aggregate or because of their organization at the cell surface (66, 67). As previously suggested (67), the non-universality of some of the capsule functions across and within species suggests that there may be some functions that are very different to those that are commonly evoked during pathogenesis. It could also be that different serotypes are associated to specific functions or fitness advantages. This might lead to selection of serotype switching when bacteria colonize novel environments. Yet, it seems more likely that the rapid

turnover of capsular serotypes is due to biotic pressures, such as phage predation, cell-to-cell interactions, the host adaptive immune system, or protozoa grazing (29, 68). Taken together, our work suggests that the primary evolutionary forces selecting for the existence of a capsule may be very different to those selecting for its subsequent diversification, and highlights that much work is still needed to fully understand the primary role of capsules, both at the molecular and at the evolutionary level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table S1. Primers used for the construction of mutants are listed in Table S3. Bacteria were grown at 37° in Luria-Bertani (LB) agar plates or in 4 mL of liquid broth under vigorous shaking (250 rpm) unless indicated otherwise. Kanamycin (50 μg/ml) or Streptomycin (100 μg/mL for *E. coli* and 200 μg/ml for *Klebsiella*) were used in the selection of the plasmids strain selection and cultures. Antibiotics were purchased from Sigma.

Growth media description. AUM and ASM stand for artificial urine medium and artificial sputum medium, respectively, and were done as described previously (41, 42). Briefly, AUM is mainly composed of urea and peptone with trace amounts of lactic acid, uric acid, creatinine and peptone. ASM is composed of mucin, DNA, egg yolk and aminoacids. LB is composed of tryptone and yeast extract. M02 and M005 indicate minimal M63B1 supplemented with 0.2% and 0.05% glucose (sole carbon source), respectively.

Determination of serotype. Complete or draft genomes were serotyped using Kaptive with default settings (69).

Natural loss of capsule. An overnight culture in LB was diluted at 1:100 in the different environments (ASM, LB, M02, M005, AUM) in a final volume of 4 mL. Cultures were allowed to grow for 24 hours at 37°C and diluted again to 1:100 in fresh media. This was repeated for 3 times. Then, each culture in each environment was serially diluted and CFUs were counted (3 plates per sample, > 100 colonies per plate). Non-capsulated mutants are easily visualized by the naked eye as mutants produce smaller, rough and translucent colonies. Results are expressed as a ratio of non-capsulated mutant colonies observed over the total plate count. The limit of detection of non-capsulated mutants in this assay was 0.22% ± 0.04 or 1:450 per biological replicate per environment. To ensure that the naturally-emerged non-capsulated colonies observed on plate are *bona fide* genetic mutants and not phase-variants, we randomly chose 10 populations, using the *sample* function in R. Three non-capsulated clones from each population were randomly selected and restreaked twice. All clones derived from the original non-capsulated colony remained non-capsulated, demonstrating that the loss of the capsule is genetic.

Generation of *Δwza* capsule mutants. Isogenic capsule mutants were constructed by an in-frame deletion of *wza* by allelic exchange. Upstream and downstream sequences of the *wza* gene (> 500pb) were amplified using Phusion Master Mix (Thermo Scientific) then joined by overlap PCR. The resulting PCR product was purified using the QIAquick Gel Extraction Kit and then cloned with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) into competent *E. coli* DH5α strain. KmR colonies were isolated and checked by PCR. Cloned Zero Blunt® TOPO® plasmid was extracted using the QIAprep Spin Miniprep Kit, and digested for 2 hours at 37°C with ApaI and SpeI restriction enzymes and ligated with T4 DNA ligase (Promega) overnight at 16° to a double-digested pKNG101 plasmid. The ligation was transformed into competent *E. coli* DH5α pir strain, and again into *E. coli* MFD λ-pir strain (70), used as a donor strain for conjugation into *Klebsiella spp*. Conjugations were performed for 24 hours at 37°C. Single cross-over mutants (transconjugants) were selected on Streptomycin plates (200 µg/ml). To select for plasmid excision, transconjugants were grown for 16 hours at 25°C and double cross-over mutants were selected on LB without salt and supplemented with 5% sucrose at room temperature. To confirm the loss of the plasmid, colonies were tested for their sensitivity to streptomycin. From each double-recombination, a mutant and a wild type were isolated. All experiments were performed in parallel with the parental strain, *Δwza* mutant and the associated wild type, to ensure that the phenotype observed was directly due to the *Δwza* deletion and not to other potential SNPs that could have accumulated in the genome during the mutant generation, and thus present in both the mutant and the wild type.

Deletion mutants were first verified by Sanger, and then checked for off-target mutations using Illumina. Three strains presented off-target mutations. Strain #63 (KL107) ***Δwza*** has a 15bp deletion upstream of *rfaH*, another capsule regulator. This inhibits the initiation of capsule synthesis altogether. Strain #51 (KL24) has a SNP in *pcaR.* There are no reports on a direct influence of this gene on capsule production. Strain #55 (KL2) has a SNP in *sacX* gene which results in delayed growth. Despite the fact that these strains are *bona fide* capsule mutants, as shown by microscopic observation and capsule quantification, the results are presented as supplemental figures.

Microscopy. Bacteria capsules were visualized using Indian Ink coloration on an exponential phase culture. Images were acquired on a Nikon Eclipse E400 camera using the Digital Net DN100 camera control unit and a 100X objective.

Capsule extraction and quantification. The bacterial capsule was extracted as described in (71) and quantified by using the uronic acid method (72). Briefly, 500 μL of an overnight culture was mixed with 100 μL of 1% Zwittergent 3-14 detergent in 100 mM citric acid (pH 2.0) and heated at 56°C for 20 minutes. After it was centrifuged for 5 min at 14,000 rpm and 300 μL of the supernatant was transferred to a new tube. Absolute ethanol was added to a final concentration of 80% and the tubes were placed on ice for 20 minutes. After a second wash with ethanol at 70%, the pellet was dried and dissolved in 250 μL of distilled water. The pellet was then incubated for 2 hours at 56°C. Polysaccharides were then quantified by measuring the amount of uronic acid. A 1,200 μL volume of 0.0125 M tetraborate in concentrated H_2SO_4 was added to 200 μL of the sample to be tested. The mixture was vigorously vortexed and heated in a boiling-water bath for 5 min. The mixture was allowed to cool, and 20 μL of 0.15% 3-hydroxydiphenol in 0.5% NaOH was added. The tubes were shaken, and 100 μL were transferred to a microtiter plate for absorbance measurements (520 nm). The uronic acid concentration in each sample was determined from a standard curve of glucuronic acid.

Competition assay. Mutants and their respective wild types were grown overnight in LB. They were then mixed in a 1:1 proportion. A sample was taken and used for serial dilution and CFU counting as a control of T_0. The co-culture was then diluted 1/100 in 4 mL of the different environments (i.e. LB, AUM, ASM, M02 and M005). After 24 hours of competition (T_{24}), each mixture was serially diluted and plated. Capsulated and non-capsulated colonies are clearly differentiated visually and counted separately. The competitive index of capsulated strains was calculated by dividing the ratio of CFU at T_{24} over T_0.

Growth curves. Overnight cultures were diluted at 1:100 in the aforementioned environments. 200 μl of each subculture was transferred in a 96-well microplate. Cell densities of cultures were estimated with a TECAN Genios™ plate reader. Absorbance (OD_{600}). Light absorbance values of each well reflect the average of 6 reads per well. Absorbance values from within-block technical replicates were averaged and these averages were used as statistically independent data points. Minimum generation times were estimated across replicates for the 1 hour interval (ΔT) spanning the fastest growth during exponential growth phase. This was calculated as:

$$\frac{1}{Td} = \frac{log_{10}(\text{maximum}_{\text{OD600}} / \text{minimum}_{\text{OD600}})}{log_{10}(2) * \Delta T}$$

Relative yields were calculated measuring ratios of the maximum absorbance of the assay compared to the reference (parental strain). The experiments were performed at least three independent times, with three technical replicates per experiment. The average of the three technical replicates per experiment was used for statistical purposes. Control experiments were performed to assess the correlation between CFU and OD_{600} during growth for both capsulated (Spearman's rho = 1.0, P = 0.002) and non-capsulated mutants (Spearman's rho = 0.94, P = 0.016). To confirm the differences in yield observed in minimal medium between capsulated and non-capsulated strains, we performed a control experiment (N = 3) in which we grew the strains #56 and #24 in microtiterplates as described before. After 16h, we plated an aliquot to count CFUs. Significantly less CFUs were observed in the non-capsulated mutants compared to wild type (P<0.05 for both strains).

Serum resistance. The ability of *Klebsiella* strains to resist killing by serum was performed as described previously (20, 43, 73). Briefly, bacteria were grown in LB to OD 600 nm of 1, pelleted by centrifugation and resuspended in sterile PBS 1X. 200 µl of bacterial suspension was added to 400 µl of pre-warmed at 37°C human sera (Sigma-Aldrich S7023), and the mixture was incubated for 2h at 37°C. Control reactions were performed with serum inactivated by heat at 56°C for 30 min. Reactions were stopped by placing on ice, and viable bacterial counts were determined before and after incubation by serial dilution and CFU plating.

Bile salts resistance. Bacteria were grown overnight in LB. As previously described (74), cultures were then serially diluted and plated in LB supplemented with either 0, 0.05% (physiological conditions) and 0.5% of CHO (primary bile salt, sodium cholate) or DCH (secondary bile salt, sodium deoxycholate). Colonies were allowed to grow and surviving CFU were counted after 24 hours. The average of two technical replicates per experiment was used for statistical purposes.

Starvation. Bacteria were grown in LB to OD=0.2, 1mL of the culture was pelleted by centrifugation and, resuspended in 400 µl of PBS 1X, as in (45). Cultures were allowed to

starve at room temperature. Aliquots were taken at 0, 30 and 60 days, and used for serial dilutions and CFU plating.

Biofilm Production. The ability of strains to form biofilm was performed as described in (75). Overnight cultures were diluted at 1:100 in the different growth media mentioned earlier. 100 µL of each diluted culture was transferred in triplicate into 96-well microplate wells and allowed to grow for 24 hours without shaking at 37°C. Cells were gently removed from the wells and washed once in distilled water. To stain biofilms, 125 µL of 1% crystal violet was added to each well for 20 minutes. The crystal violet was gently decanted and each well was washed three times with distilled water. After the plate was totally dry, the biofilm was solubilized for 10 min in 150 µL of mix with 80% ethanol and 20% acetone. The absorbance of the sample was read at OD_{570}. At least 3 biological independent replicates were performed for each sample.

Hypermucoviscosity. The optical density OD_{600} of overnight cultures were first determined by spectrophotometry as described in (43). The cultures were then sedimented by slow centrifugation (at 2,500 X g) for 5 min at room temperature. The optical density OD_{600} of the top 650 µL of supernatant was determined. Results were expressed as a ratio of the supernatant OD_{600} to that of the input culture.

Dessication test. Dessication assay was carried as previously described in (76). Briefly, 25 uL of exponentially-growing *Klebsiella* strains were deposited at the bottom of microtiterplates, and allowed the culture to dry under a laminar flow hood. Strains were then left either at 37° for one, two or four days or at room temperature for two, four, seven and 14 days. PBS was added to resuspend the cultures and diluted serially from CFU counts.

Quantification of dead cells. Cells were allowed to grow as described above with a minor modification; over day cultures were used to inoculate the 96-well microtiter plates. After 16 hours of growth in M02, dead cells were quantified using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit from Invitrogen, following provider instructions. Images were acquired on a Nikon Eclipse E400 microscope using the Digital Net DN100 camera control unit and a 100X objective. Cells were counted using ImageJ software (https://imagej.net/). On average, 6 pictures were taken per sample (min = 3, max = 10) and *ca.* 550 cells per sample were observed (min = 161, max =1604). The experiment was performed four times.

Growth on exogenously added capsule. Capsules were extracted from either wild type strains or their respective Δ*wcaJ* or Δ*wbaP* (for strain 24) mutants and quantified as described above. To initiate growth, overnight cultures of Δ*wcaJ* mutants were diluted at 1:100 in M63B1 media. Media was supplemented with either 0.8 ng/uL of extracted capsule from wild type or an equivalent volume extracted from the Δ*wcaJ* or Δ*wbaP* (for strain 24) mutants. As a negative control, cultures were allowed to grow in M63B1 with no nutrients. In parallel, M63B1 was supplemented with glucuronic acid (the standard used for estimating capsule concentration) at 0.8 ng/uL final. Growth was allowed for 48 hours at 37° and 250 rpm. Bacterial counts were performed at $T_{0,}$ T_{24} and T_{48} by serial dilution and plating. Results are expressed as the increase in CFU over 24h.

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FIGURE LEGENDS

Figure 1. Frequency of capsulated colonies across strains and growth media after three days of evolution. Strains were diluted 1:100 and inoculated in fresh media every 24 hours during three days. Strain #212 was unable to grow in M005. Points represent the average loss of capsule across at least three independently evolving populations per strain. Error bars represent standard deviation across replicate populations.

Figure 2. Competition between capsule mutants and their associated wild type in different growth media. Each point represents the average of at least three independent replicates. Individual error bars for each strain are not displayed for clarity purposes. Colours represent different serotypes (as in Figure 1), whereas the shape represent different species. Results for the strains with compensatory mutations are presented in the supplemental material (Figure S5). * $P < 0.05$; ** $P< 0.01$, Tukey post hoc and correction for multiple tests.

Figure 3. **Survival, growth rate and yield of strains in each environment. A**. Survival of parental, *Δwza* mutant and the associated wild type, relative to day 0. Data is $\log_{10}$ -transformed. Individual error bars for each strain are not displayed for clarity purposes. Colours represent different serotypes (as in Figure 1), whereas the shape represent different species (ANOVA, $P > 0.05$). **B.** Minimum generation time of each strain in each environment, expressed in minutes. Error bars represent standard deviations of the mean, of at least three independent experiments. **C.** Maximum yield of the mutant and the wild type strains relative to the parental strain in each environment. Statistics were calculated using a paired t-test. * $P < 0.05$.

Figure 4. Fate of capsule mutants in host-related context. A. Resistance to human serum. Data is presented as relative survival to control (heat-inactivated human serum). * $P< 0.05$; ** $P< 0.01$; *** $P<0.001$ paired t-test. **B.** Correlation between the total amount of biofilm formed by the wild type and the difference in biofilm formation between the wild type and the deletion mutant across. P-values correspond to Spearman's rank correlation. Results are

qualitatively similar when only taking into account the five strains with no off-target mutations. Absolute values of biofilm formation are shown in Figure S8.

Figure 5. The environment, not the serotype, explains most of the variance observed across capsule-associated traits. A.PCA analyses of capsule-related phenotypes. Phenotypes are coloured by environment. Each individual point represents a strain. PCA was performed using *prcomp* function (options scale and center = TRUE) in R and ggbiplot package for visualization purposes. **B.** Correlation matrix of all traits. Correlation matrix of all traits. Numbers indicate Spearman's correlation. Stars identify significant correlations (P<0.05).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Capsule quantification of each strain in each growth media by the uronic acid method.

Figure S2. Ratio of capsulated colonies throughout 30 days of evolution in two media. Strains were diluted 1:100 and inoculated in fresh media every 24 hours during 30 days. CFUs were counted every day. Each line represents an independent population (N = 3).

Figure S3. Capsule quantification of each mutant by the uronic acid method. This experiment was performed on overnight cultures grown in LB. Each dot corresponds to an independent experiment. The background noise of this assay is established around 1.5 ng/uL. Statistical tests: * $P < 0.05$; ** $P < 0.01$; *** $P<0.001$.

Figure S4. Fitness advantage/disadvantage of capsulated strains in nutrient poor/rich media. Competition between wild type and their isogenic capsule mutants in different growth media. Each point represents the average of at least three independent replicates. * $P < 0.05$ Tukey post hoc, p-values adjusted for multiple testing.

Figure S5. **Survival of strains under starvation and desiccation. A**. Survival of parental, mutant and wild type strains, relative to day 0. Data is $\log_{10}$ -transformed. Error bars are not displayed for clarity purposes. Colours represent different serotypes (as in Figure 1), whereas the shape represent different species. **B.** Cellular survival, in CFU, after 1, 2 or four days of desiccation at 37°C. Error bars for independent points are not shown for clarity purposes.

Figure S6. **Growth rate and yield of strains in each media. A.** Minimum generation time of each strain in each media, expressed in minutes. Error bars represent standard deviations of the mean, of at least three independent experiments. **B.** Maximum yield of the mutant and the wild type strains relative to the parental strain in each growth media. Statistics were calculated using a paired t-test. * $P < 0.05$. **C.** Percentage of dead cells after 16 hours growth in a microtiter plate well. Each symbol represents an independent experiment. No significant differences are found across genotypes.

Figure S7. **Log_{10}-Increase of CFU after 48 hours of growth.** Growth of *Δ*wcaJ (26 and 56) and *Δ*wbaP (24) mutants was followed for 24 and 48 hours. Cells were grown in M63B1 without nutrient source (Control), with 0.8 ng/uL of purified capsule (Cap+), an equivalent volume of extraction of a non-capsulated strain (Cap-), and 0.8 ng/uL of glucuronic acid (the standard used to quantify the capsule). Individual points represent four independent experiments.

Figure S8. Fate of capsule mutants in host-related context. **A.** Resistance to human serum. Data is presented as relative survival to control (heat-inactivated human serum). **B.** Resistance to bile salts at physiological concentrations (0.05%), and at 10X (0.5%). Results are presented as Log_{10} (relative to control, LB plates). * $P < 0.05$, ** $P< 0.01$, *** $P < 0.001$

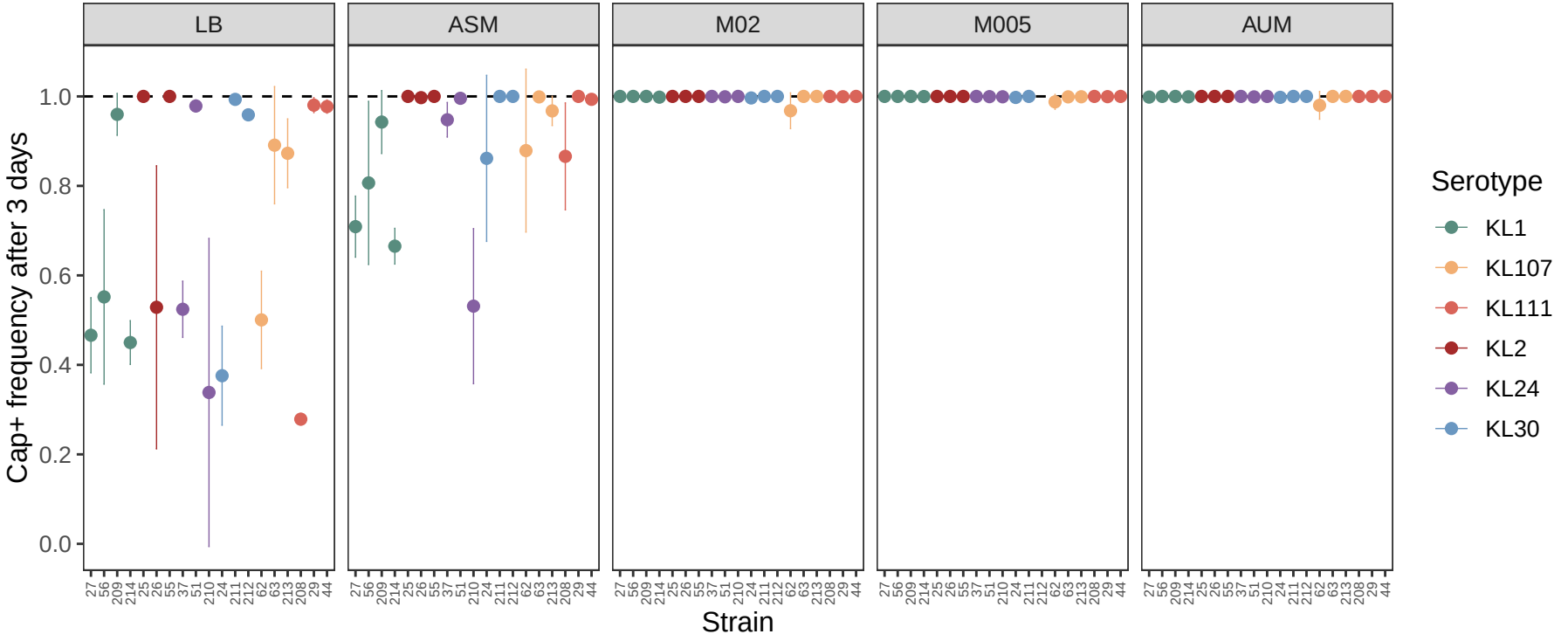
Figure S9. Biofilm formation of parental, mutant and wild type strains across growth media. P values reflect paired t-tests. * $P < 0.05$, ** $P< 0.01$, *** $P < 0.001$

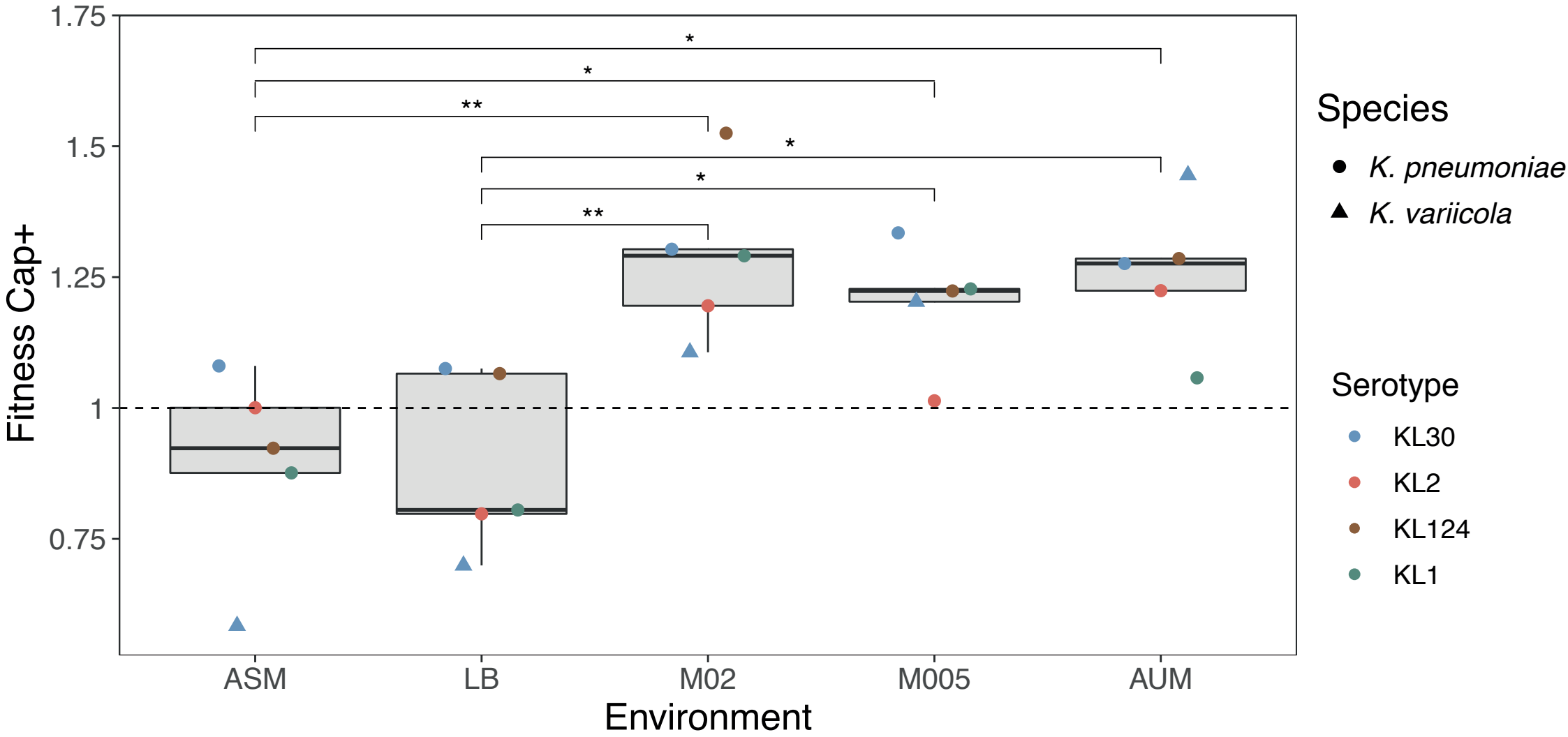
Figure S10. Growth rate and maximum yield of each population in each environment.

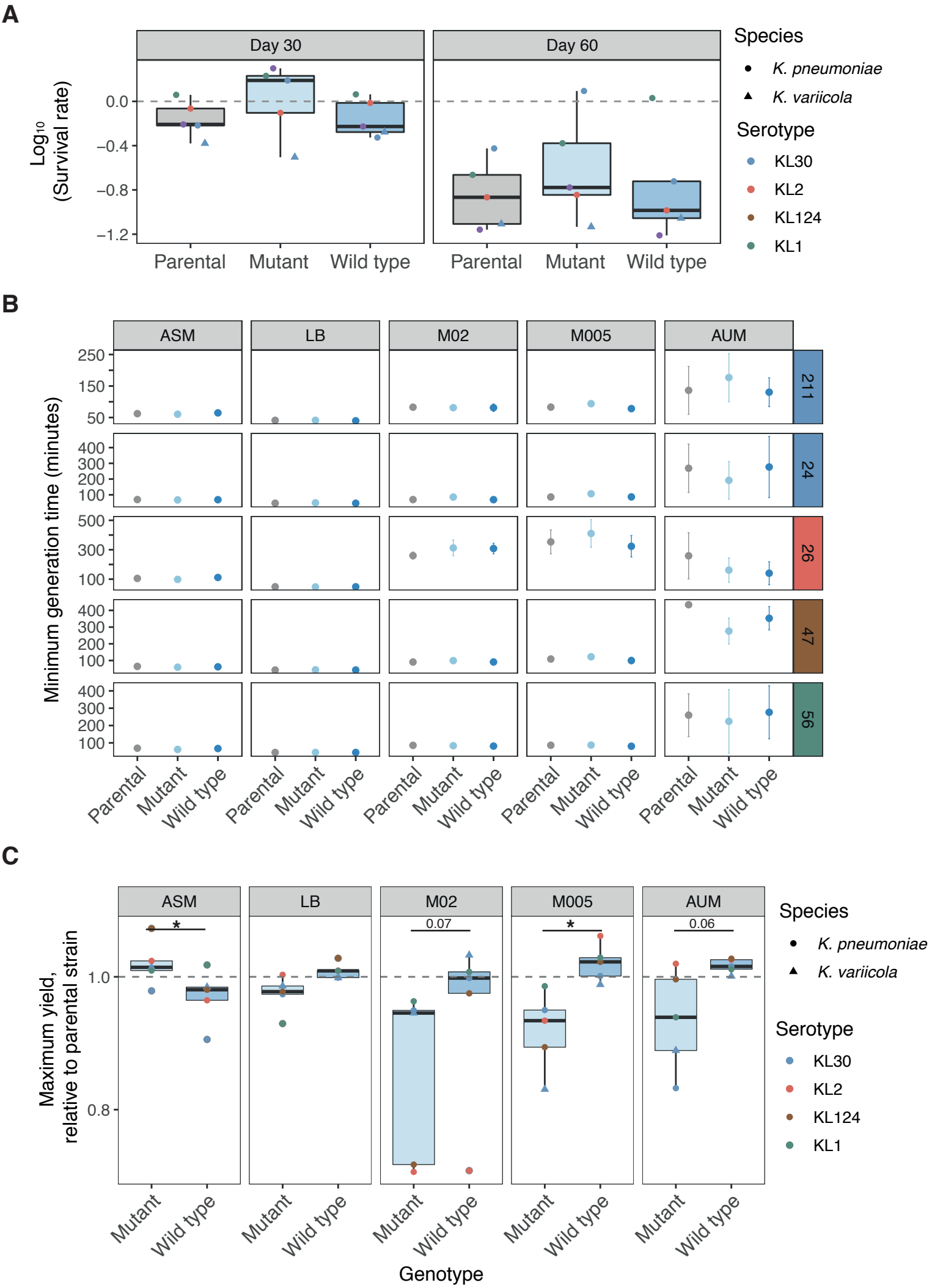
Figure S11. Biofilm formation of all strains in all environments.

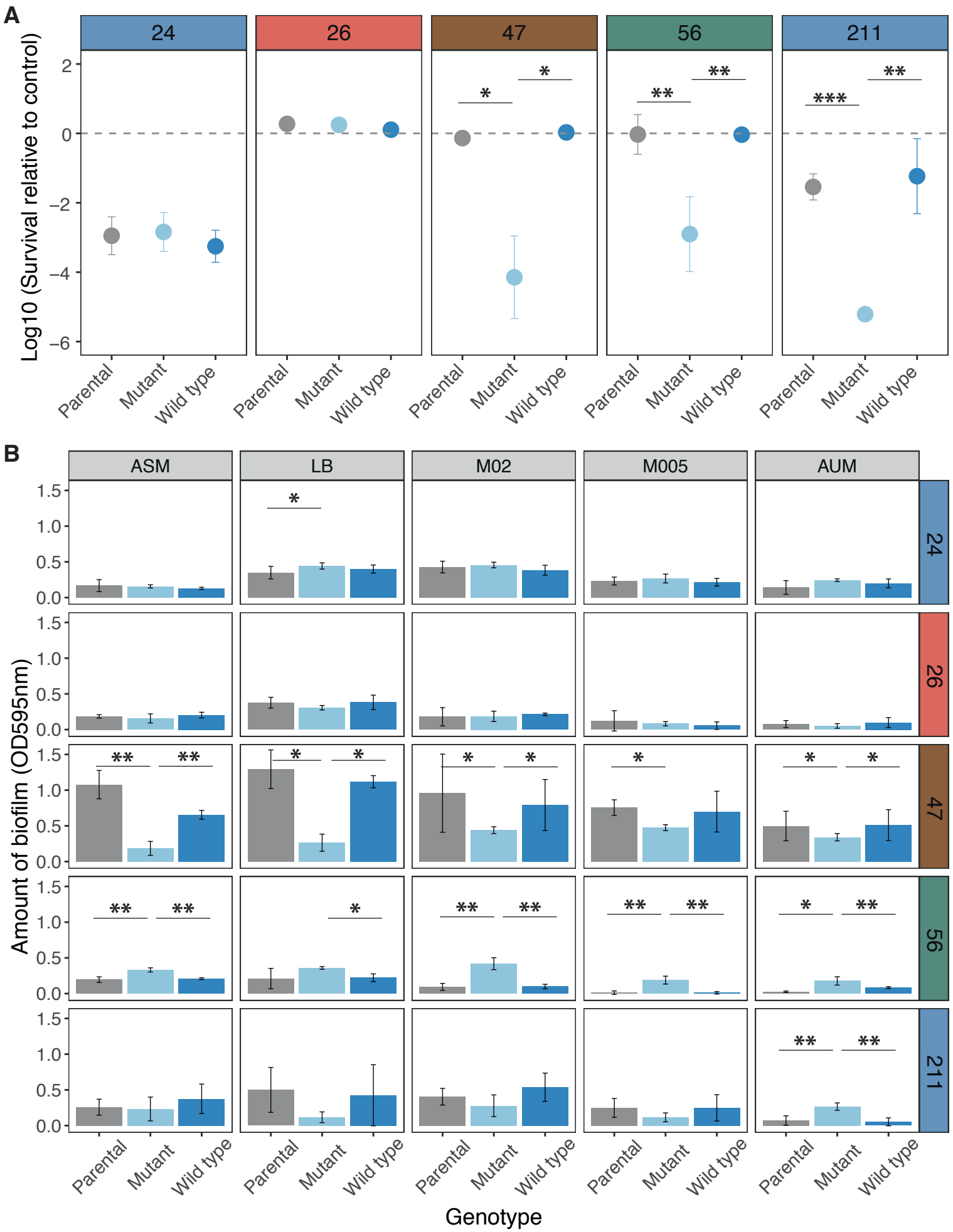
Figure S12.Mucoviscosity index of strains in all environments.

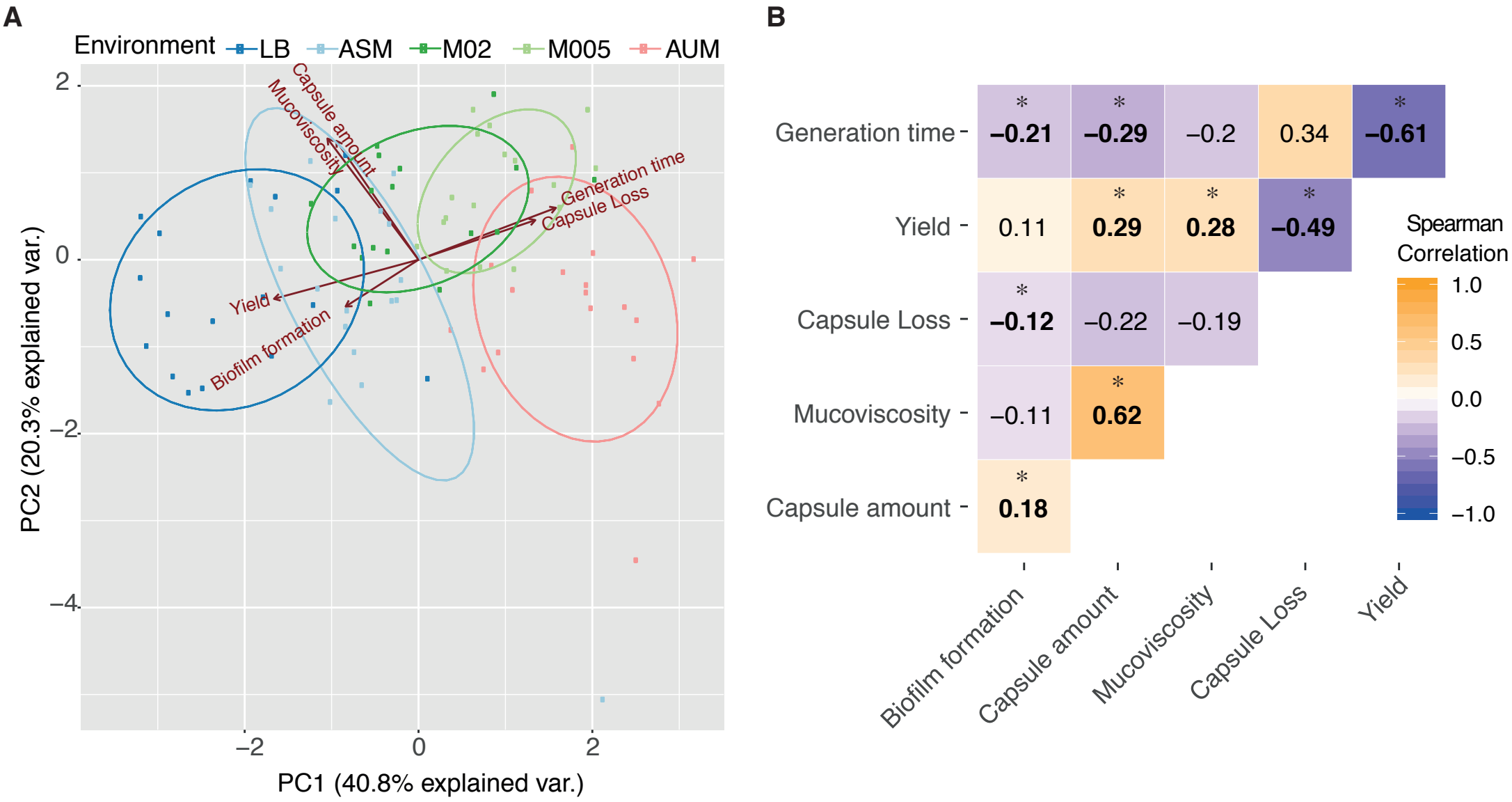
Figure S13. PCA analyses of capsule-related phenotypes. Phenotypes are coloured by serotype. Each individual point represents a strain. PCA was performed using *prcomp* function in R and ggbiplot package for visualization purposes.


LB
ASM
M02
M005
AUM
Cap+ frequency after 3 days
1.0
0.8
0.6
0.4
0.2
0.0
27 56 209 214 25 26 55 37 51 210 24 211 212 62 63 213 208 29 44
Strain
Serotype
KL1
KL107
KL111
KL2
KL24
KL30


1.75
1.5
1.25
1
0.75
Fitness Cap+
*
*
**
*
*
**
ASM
LB
M02
M005
AUM
Environment
Species
K. pneumoniae
K. variicola
Serotype
KL30
KL2
KL124
KL1


A
Day 30
Day 60
Log10 (Survival rate)
0.0
−0.4
−0.8
−1.2
Parental
Mutant
Wild type
Species
K. pneumoniae
K. variicola
Serotype
KL30
KL2
KL124
KL1
B
ASM
LB
M02
M005
AUM
Minimum generation time (minutes)
250
150
50
400
300
200
100
500
211
24
26
47
56
C
Maximum yield, relative to parental strain
1.0
0.8
*
0.07
0.06
Genotype


A
24
26
47
56
211
Log10 (Survival relative to control)
2
0
−2
−4
−6
*
*
**
**
***
**
Parental
Mutant
Wild type
B
ASM
LB
M02
M005
AUM
Amount of biofilm (OD595nm)
1.5
1.0
0.5
0.0
Genotype


A
Environment
LB
ASM
M02
M005
AUM
Capsule amount
Mucoviscosity
Generation time
Capsule Loss
Yield
Biofilm formation
PC2 (20.3% explained var.)
PC1 (40.8% explained var.)
2
0
−2
−4
−2
0
2
B
Generation time
Yield
Capsule Loss
Mucoviscosity
Capsule amount
Biofilm formation
Capsule amount
Mucoviscosity
Capsule Loss
Yield
* −0.21
* −0.29
−0.2
0.34
* −0.61
0.11
* 0.29
* 0.28
* −0.49
* −0.12
−0.22
−0.19
−0.11
* 0.62
* 0.18
Spearman Correlation
1.0
0.5
0.0
−0.5
−1.0