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Research Article

THE PREFERENCE PRIORITY OF *Bacillus subtilis* IN UPTAKING FREE DNA DURING THE NATURAL TRANSFORMATION

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Abstract: Although genetic material is vertically transferred between generations via sexual or asexual reproduction, similarities in some chromosome and gene parts of unrelated organisms provide important clues for another way of transfer. The mobility of genetic information among different organisms, known as horizontal gene transfer (HGT) has immediate or delayed effects on the recipient host. One of the most notable mechanisms of HGT is natural transformation (NT), a process in which cells take free DNA from the extracellular environment and incorporate it into their chromosomes by homologous recombination. NT is widely conserved in many bacterial species as it can promote to spread of resistance genes. Although it is known that many organisms rely on HGT, there is limited information about how they decide which particular genetic material to horizontally transfer.

Here, I have investigated the preference priority among different gene sources presented under certain stress conditions for *Bacillus subtilis* possessing NT ability. To test this, two DNA specimens (E and C) with different sequence contents of the same length were presented to *B. subtilis* under different stress environments (BK, BC, BE and BCE). The hypothesis was evaluated according to the analysis of the results of colonial formations on selective plates (pE, pC and pCE). The obtained data presented a strong positive correlation that the bacteria have preference priority during NT depending on a stimulator. The tendency of the bacteria to uptake useful DNA fragments in a specific environment can be suggested. For instance, the majority of colonies grow on pE plates rather than the pC and pCE when the transformation media includes erythromycin (Eryt) as an inducer. Although the data significantly overlaps with the idea claiming that the bacteria have a preference priority to uptake free DNAs during NT, further investigations are needed to support the present data and for better understanding of the phenomenon.

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Key words:
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Özet: Genetik materyal nesiller arasında genellikle eşeyli veya eşeysiz üreme yoluyla dikey olarak aktarılsa da, akraba olmayan organizmaların bazı kromozom ve gen kısımlarındaki büyük benzerlikler, başka bir aktarım yolu olabileceğini gösterir. Farklı organizmalar arasında genetik bilginin hareketliliği olarak bilinen yatay gen transferi (YGT), alıcı konak üzerinde ani veya gecikmeli etkilere sahiptir. YGT'nin en dikkate değer mekanizmalarından biri, hücrelerin hücre dışı ortamdan serbest DNA aldığı ve homolog rekombinasyon yoluyla kromozomlarına dâhil ettiği bir süreç olan doğal transformasyondur (DT). DT, direnç genlerinin yayılmasını teşvik edebildiği için birçok bakteri türünde yaygın olarak korunur. Birçok organizmanın YGT gerçekleştirdiği bilinmesine rağmen, organizmaların yatay olarak aktarılan genetik materyale nasıl karar verdiği hakkındaki bilgi sınırlıdır.

Burada, DT yeteneğine sahip *Bacillus subtilis*'in belirli stres koşulları altında sunulan farklı gen kaynakları arasından seçim önceliğini araştırdım. Bunu test etmek için, aynı uzunlukta fakat farklı dizi içeriğine sahip iki DNA örneği (E ve C), farklı stres ortamları (BK, BC, BE ve BCE) altında *B. subtilis*'e sunuldu. Hipotez, DT sonrasında seçici plakalar (pE, pC ve pCE) üzerinde oluşan kolonilerin analiz sonuçlarına göre değerlendirildi.

Elde edilen veriler, DT sırasında bakterilerin bir uyarıcıya bağlı olarak tercih önceliğine sahip olduğuna dair güçlü bir pozitif korelasyon sunmuştur. Bakterilerin belirli bir ortamda yararlı DNA parçalarını alma eğilimi gösterdiği söylenebilir, örneğin, dönüştürme ortamı bir indükleyici olarak erythromycin (Eryt) içerdiğinde kolonilerin çoğunluğu pC ve pCE yerine pE plakaları üzerinde büyümüştür. Veriler, bakterilerin DT sırasında serbest DNA'ları almak için bir tercih önceliğine sahip olduğu iddiasıyla önemli ölçüde örtüşse de, verileri güçlü bir şekilde desteklemek ve fenomeni doğru bir şekilde anlamak için daha fazla araştırmaya ihtiyaç vardır.

Introduction

Genetic material is generally vertically transferred from parental lines to offspring in almost all species via sexual or asexual reproduction. However, huge similarities in some chromosomes and gene parts of various organisms that are not evolutionarily related to each other provide important clues that genetic material transmission between these species may occur in different ways. In particular, comparative genome analyses of prokaryotes associate the evolutionary change of these organisms with genetic materials shared in horizontal ways. For example, a study comparing 88 different prokaryotic genomes revealed that there is a 0-22% genome exchange between bacteria and 5-15% in archaea by horizontal gene transfer (HGT) (Garcia-Vallve 2003). Bacteria share genetic material among themselves by means of conjugation, transduction and transformation (Lorenz & Wackernagel 1994, Luo & Wasserfallen 2001). In this way, the host bacterium can acquire a new feature that it did not have before and become more adaptable to its environment.

HGT provides genetic mobility between different organisms to form an immediate or postdated impact on the recipient host (Husnik & McCutcheon 2016). Transformation, transduction and conjugation are the major natural mechanisms that mediate HGT. HGT also occurs through gene transfer agents, nanotubes and exosomes (Singorenko *et al*. 2017, Hong *et al*. 2019). HGT-mediated DNA recombination integrates DNA directly obtained from a donor or environment into the genome of the recipient cell. This phenomenon is responsible for the transfer of mobile genetic elements (MGEs) such as transposons, integrons and/or gene cassettes between bacterial species and extensively occurs in the bacterial kingdom. Although HGT is more common between two bacterial species (particularly in archaea and bacterial groups), it also frequently occurs between a donor bacteria and other organisms such as fungi, plants, and animals that act as recipients (Garcia-Vallve *et al*. 2000, Rancurel *et al*. 2017).

HGT can provide cross-genetic transitions between two relatively distant species. Phylogenetic studies have shown that approximately 145 foreign genes were incorporated into the human genome throughout the evolutionary process (Crisp *et al.* 2015). Most of these genes have been associated with foreign mitochondrial genes and retroviral vectors (Salzberg 2017). HGT was found to make contribution in the evolution of the bacterial genome, prevention of genome damage, antibiotic resistance and adaptability to environmental stresses (Hall Brockhurst & Harrison 2017). HGT usually occurs among taxa in a closed environment. In conditions where donor-recipient genomes are in close contact with each other, the probability of occurrence of HGT increases (Adato *et al*. 2015, Brown & Wernegreen 2019). According to phylogenetic and eukaryotic nuclear genome analysis, the expression of genetic information carried by HGT could affect a wide variety of genes (Baquero *et al.* 2019, Campos *et al*. 2019, Leclerc *et al*. 2019). Therefore, it can be said that HGT plays an effective role in the evolution of both prokaryotic and eukaryotic genomes.

One of the most notable mechanisms of HGT is natural transformation (NT), a process in which cells take free DNA from the extracellular environment and incorporate it into their chromosomes by homologous recombination or reassemble it as part of the self-replicating episome (Blokesch 2016). NT is an evolutionarily conserved and phylogenetically widespread mechanism (Johnston *et al.* 2014) and can mediate the acquisition of large DNA fragments (7-50 kb) (Blokesch 2017).

NT is identified in naturally competent bacteria by two sequential steps; the physiologically regulated uptake of exogenous free DNA and its genomic integration (Nielsen & van Elsas 2019). About 130 species in almost all groups of the bacterial kingdom, including soil bacteria and many important human pathogens, have been characterized by their ability to perform NT under naturally-occurring environmental conditions (Johnston *et al.* 2014, Kuffner *et al.* 2015). The DNA uptake with the NT process either uses different species-specific uptake sequences or is sequence-independent (Nielsen & van Elsas 2019). The efficiency of transformation due to homologous recombination depends on the sequence similarity between the imported linear DNA and the recipient genome (Lorenz & Wackernagel 1994).

NT is widely conserved in many bacterial species as it can promote the acquisition and spread of antibiotic resistance genes (ARG) and virulence factors (Lorenz & Wackernagel 1994, Blokesch 2016). Although 90 years have passed since the discovery of NT (Griffith 1928), many aspects of the process remain unclear.

Cells generally uptake double-stranded DNA (dsDNA) from the medium during NT, but only one strand can reach out to the cytoplasm (Lacks 1962, Gabor & Hotchkiss 1966, Piechowska & Fox 1971). The single-strand DNA (ssDNA) is rapidly bound by DprA and the recombination protein RecA just after the entrance to the cytoplasm (Kidane & Graumann 2005, Mortier-Barrière *et al.* 2007). Previous studies have shown the molecular evidence suggesting that a ssDNA is directly integrated into the genome (Fox & Allen 1964, Dubnau & Davidoff-Abelson 1971, Mejean & Claverys 1984). Thus, the ssDNA-RecA complex probably first performs homologous sequence scanning on the genome. Then RecA promotes a D-loop formation by strand invasion where sequence similarity exists. Subsequently, the branches of the D-loop are likely elongated by a process known as branch migration (NT-specific branch migration factors have been identified) (Marie *et al.* 2017, Nero *et al.* 2018). Following branch migration, this three-stranded complex is assumed to be processed by a mechanism (not completely understood yet) that creates a stable heteroduplex structure between the free ssDNA and the genome. Although it is known that the repair mechanisms play an important role to untie the

heteroduplex structure and completing genome recombination, the whole mechanism is understudied. To summarize, although it is known that HGT is carried out by many organisms (Burmeister 2015, Tenlen *et al.* 2016, Arnold *et al*. 2021), there is limited information about how organisms select or determine a certain genetic material horizontally transferred.

Here, we have investigated the preference priority among different gene sources presented under certain stress conditions for *Bacillus subtilis* possessing NT ability. To test this, two DNA specimens with different sequence contents of the same length were presented to *B. subtilis* under different stress environments. To understand whether the NT mechanism has a conscious preference to pick DNA, the tendency of *B. subtilis* to uptake a certain free DNA has been examined in terms of whether the preferred DNA enables any benefit for disadvantaged conditions.

Materials and Methods

Strains and strain cultivation

The plasmids used in this study were amplified in *Escherichia coli* DH5α strain. *B. subtilis* W168 was used to test the NT mechanism. Both bacteria were grown in Luria - Bertani (LB) broth medium (1L prepared from 10 g tryptone, 5 g NaCl and 10 g Yeast extract) at 37°C at 180 rpm. Since *B. subtilis* is capable of NT under favourable conditions, a specific culture medium (MNGE) was used to provide the transformation conditions (Supplementary Material Table 1).

For short-term storage, *E. coli* was saved at +4°C, while *B. subtilis* is kept at room conditions on an LB agar plate (cold conditions such as +4°C can be fatal). Long-term stocks including LB culture collected in the logarithmic phase and 20% glycerol solution were kept at -80°C.

DNA sources

pBS1C (C) and pBS2E (E) are transformation vectors developed for gene transfer processes into *B. subtilis* (Radeck *et al.* 2013) (Supplementary Material Table 2). Both C and E are the basic shuttle vectors that can be used for both *E. coli* and *B. subtilis* transformation. Although the vectors share some common genetic parts such as *E. coli* replication origin (ori) for -amplification and ampicillin-resistant gene (*bla*) for *E. coli* colony selection, they have different antibiotic-resistant genes for *B. subtilis* colony selection; C carries chloramphenicol (Chl) resistant gene (*cat*), while E has erythromycin (Eryt) resistant gene (erm).

Both vectors are integrative plasmid for *B. subtilis*; E contains Eryt gene between two homologous sequences for endogenous *lacA* gene, coding for β-galactosidase, and C has Chl gene between the homologous sequences of endogenous *amyE* - alpha-amylase. These vectors are used as basic bricks to transfer desired genes to *B. subtilis* genome via homolog recombination. Here we used their linearized form to integrate antibiotic resistant genes (*cat* and *erm*) to *B. subtilis* genome.

Vector transfer to *E. coli* was performed according to the protocol of Sambrook & Russell (2006). Positive colonies selected on LB agar medium containing ampicillin were grown in liquid LB medium at 37°C at 180 rpm until OD595 reach 2-3. Plasmid isolations were performed following the GeneJET Plasmid Miniprep Kit (Thermo-Fisher, USA) protocol. Plasmid concentrations were measured by NanoDrop 2000/2000c (Thermo, USA).

Experimental design to test NT mechanism in B. subtilis

The transformation experiment was designed to test the major objective of the study is detailed in Fig. 1. The preference priority of *B. subtilis* to uptake free DNA among two specimens in the medium via HGT was tested. Two DNA fragments of approximately the same size (C and E) were used for the experiments. *Bacillus subtilis* is able to uptake DNA fragments (preferably linear, if the plasmid cannot replicate) from the culture medium via NT and successfully integrate them into its genome by homologous recombination (Radeck *et al.* 2013). Both E and C were linearized by cutting with appropriate restriction enzymes (*ScaI*) due to the lack of the replication origin for *B. subtilis* (Fig. 1c).

Four different transformation culture media were prepared for the experiments: BK, BC, BE and BCE. Both linearized DNA fragments, C and E, were added into all media, at least 100 ng each. By doing so, two different DNA options were provided to bacteria to choose from in each cultural condition. To test how stress factors would influence the tendency of *B. subtilis* to select the DNA fragment in the medium, BC, BE and BCE media were prepared with various stress ingredients. The very low/non-lethal dose of antibiotics that had already been tested on cell viability was used to only induce bacteria. 50 ng Chl (5 ng/ml) was added as an inducer to the BC medium, 10 ng Eryt (1 ng/ml) to the BE medium, and both 50 ng Chl and 10 ng Eryt to the BCE medium. No stress component was added to the control environment BK (Fig. 1d).

Bacillus subtilis cultured in BK, BC, BE and BCE to occur NT according to the protocol detailed in Supplementary Data, Table 3. Subsequently, to obtain colonies, each of the BK, BC, BE and BCE cultures were spread on three different selective media: pE (LB agar with 1 µg/ml Erythromycin), pC (LB agar with 5 µg/ml Chloramphenicol) and pCE (LB agar with 1 µg/ml Erythromycin and 5 µg/ml) (Fig. 1b).

NT conditions for B. subtilis

Here we briefly explain the NT transformation protocol for *B. subtilis*. The fresh bacterial cultures were taken at logarithmic phase of LB culture to inoculate in the rate of 0.1 OD_{600} into 10 ml MNGE (Supplementary Material Table 1) and cultivated overnight at 37°C, 200 rpm until 1.1-1.3 OD_{600}. Then, 400 µl of cell suspension was transferred into four 15 ml test tubes (BK, BC, BE and BCE) for transformation. Subsequently, 1-2 µg linear DNA of E and C were added in each media before culturing them at 37°C, 200 rpm for 1 hour.

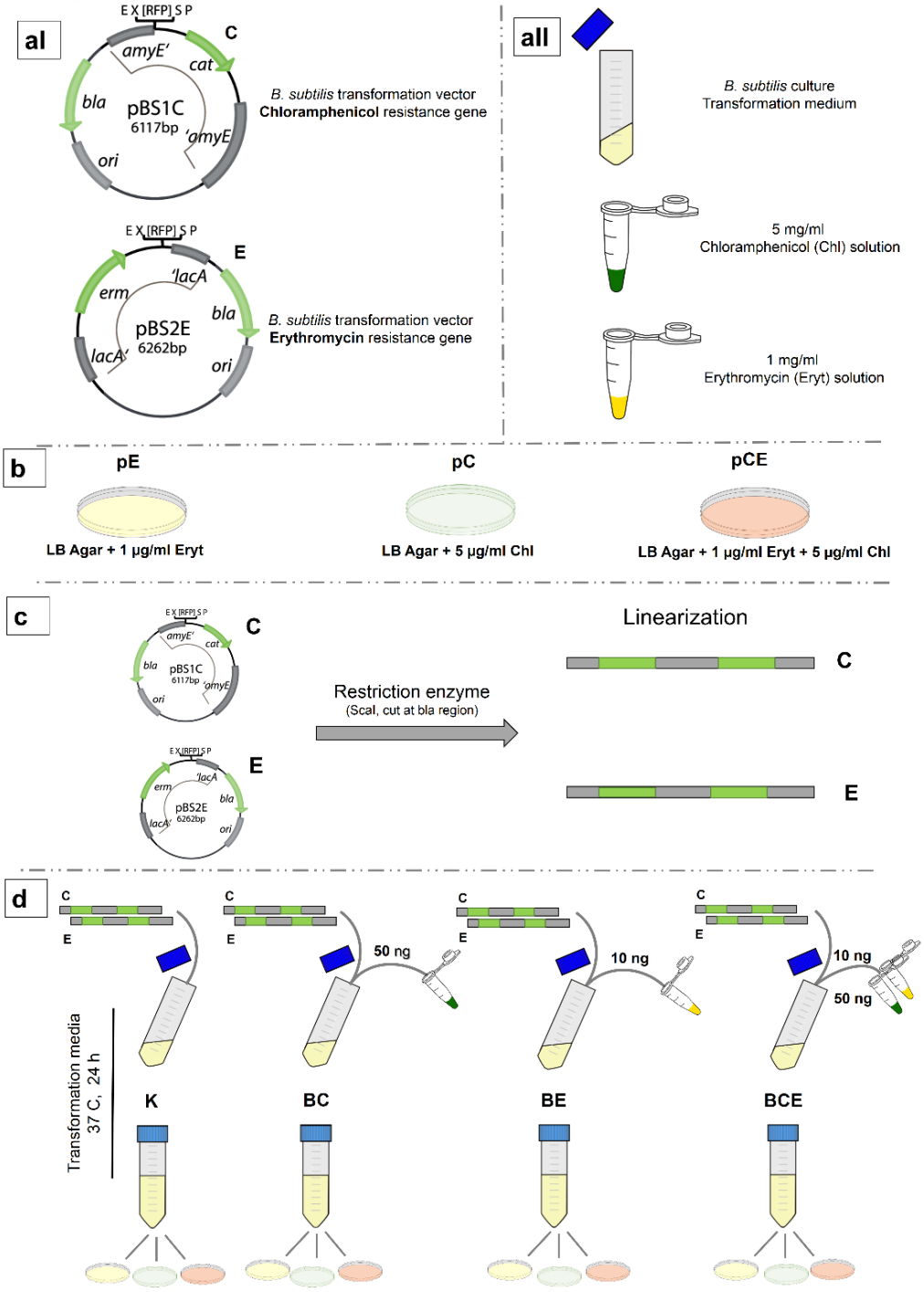

Fig. 1. The preference priority of *B. subtilis* when uptaking free DNA among different options with NT under particular circumstances. **aI.** The presented DNA options for transformation, the vectors developed for gene transfer to *B. subtilis*; pBS1C (resistance gene "*cat*" for Chloramphenicol (Chl), *AmyE* sequences for homologous recombination into *B. subtilis* genome, red fluorescent protein (RFP), plasmid replication origin for *E. coli* (ori) and ampicillin resistance gene (*bla*) for *E. coli* selection) and PBS2E (resistance gene "*erm*" for Erythromycin (Eryt), l*acA* sequences for homologous recombination into *B. subtilis* genome, red fluorescent protein (RFP), plasmid replication origin for *E. coli* (ori) and ampicillin resistance gene (*bla*) for *E. coli* selection), **aII.** *Bacillus subtilis* transformation medium, 5 mg/ml Chl solution and 1 mg/ml Eryt solution, **b.** selective LB agar plates: pBE; LB agar containing 1 µg/ml Eryt pBC; LB agar including 5 µg/ml Chl; pBCE; LB agar containing both 1 µg/ml Eryt and 5 µg/ml Chl, **c.** vectors were linearized by cutting with *ScaI* from the bla gene region, **d.** linearized C and E plasmids were included in all MNGE transformation media: BK; control environment, no stress inducer. BC; including 50 ng Chl as a source of stress reminder. BE; containing 10 ng Eryt as a stress trigger. BCE; harbouring both 50 ng Chl and 10 ng Eryt as stress inducers. After the transformation process, the cells from each culture were separately spread on pBE, pBC and pBCE selective media for colony analysis.

After that, 100 µl expression mix (Supplementary Material Table 1) was added in each of them and cultured at 37°C, 200 rpm for 1 hour. Lastly, 50 µl of bacterial cultures taken from each media were spread on the selective solid agar plates (pE, pC and pCE).

Colony assay

The transformation success of *B. subtilis* cultures in BK, BC, BE and BCE were evaluated in selective LB agar plates. Samples from each culture medium were spread on three different selective media, pE, pC and pCE. Formed colonies were counted and photographed. The colonies were analysed by PCR to verify the genome integration of linear DNAs.

Results and Discussion

In this study, the preference priority of *B. subtilis* W168 strain for uptaking free DNA from culture environments during the HGT process was investigated. Cultivation media were prepared with two linearized fragments of plasmid DNAs (C and E) to provide selection options to the bacteria. Both integrative DNA fragments are approximately equal in length and each of them contains one+ different antibiotic resistance gene (C has *"cat"* gene for Chl, and E has *"erm"* gene for Eryt). *"cat"* and *"erm"* can easily integrate into *B. subtilis* genome owing to homologous sequences of some endogenous genes flanked to both sides of the genes (Radeck et al., 2013). Here we investigated whether the bacteria have any tendency to pick a certain DNA over the other option under certain environmental conditions.

Four transformation media (BK, BE, BC and BCE) were prepared to test the research question. Equal amounts of both linearized DNA fragments (C and E) were added to all media. Low doses of Chl or/and Eryt antibiotics were added into three culture environments except for the control to assess whether the bacteria display any preference priority for certain DNA fragments that could enable them to gain an advantage against associated stress conditions. After the transformation process was completed, an equal volume of bacterial community from each medium was inoculated into three different selective solid media (pE, pC and pCE) (Fig. 2).

When the colonies formed by bacteria planted in selective solid plates from the BK culture (control group) are considered, it is apparent that the difference between the numbers of colonies (±104) on pC is significantly different from pE (±6) and pCE (±1) which have almost no colony. It was observed that the bacteria mostly uptakes the C fragment rather than neither E nor C and E together in the absence of any inductive agent (Figs 2-3a).

Although the colony outputs of the BC group have a similar pattern to the control group, the number of colonies formed by the BC group is 2-fold of the BK (Fig. 3). The addition of Chl into the transformation media resulted in numerous colonies on pC (±199), but very few numbers on pE (±15) and pCE (±20). At least, the BC group has more colonies on pE and pCE in comparison to the plates of the BK group. In that point, it is hard to make a conclusion on whether the addition of Chl has induced the bacteria to uptake specifically the C fragment due to the insignificant differences between the outputs of BK and BC groups. However, it is clear that the presence of a provoker agent in the transformation media has increased the efficiency of NT, regarding these results (Fig. 3b).

The greater part of colonies popped up on the pE (±548) solid media when Eryt was added to the transformation medium (BE) to stimulate the bacteria (Figs 2, 3). Contrary, there are a couple of colonies on the pC (±26) and pCE (±8). Colonies planted from BE medium mostly have fragment E compared to BK and BC-based ones. It seems that the presence of Eryt in the medium has induced the bacteria to uptake fragment E rather than other options. Despite the similarity between BC and BK-driven results, the results of BE and BC groups can make us think that the low level of a definite stress factor stimulates the bacterial NT to uptake a certain free DNA among several other options. Thus, the relevant DNA source can enable them an advantage to deal with a certain stress condition. For example, here the bacterial NT was induced with Eryt, and the majority of colonies have E fragments including a resistance gene against Eryt rather than other DNA options (C or C+E) in the culture (Figs 2, 3).

The bacteria stimulated in the medium (BCE) including both Eryt and Chl antibiotics as inducers have mainly formed colonies on the pE (±201) and the pCE (±124) plates, and very few on the pC (±24). The pCE plates planted from BCE culture have more colony numbers compared to BE-driven pCE ones (Fig. 3). The stimulation of the transformation medium with both Eryt and Chl might have increased the uptake of E and C fragments together by the bacteria. This has provided them to grow on the selective pCE plates including both Eryt and Chl. In comparison to other plates, pCE ones contain colonies in petite formation (Fig. 2). High-stress conditions mostly force organisms to grow in a smaller metabolic structure (Soufo 2016, Vowinckel *et al.* 2021). Hence, it is normal to see petite colonies on the pCE dishes including two selective agents.

When all results are considered together, a strong positive correlation that the bacteria have preference priority during NT depending on a stimulator is apparent. The tendency of the bacteria to uptake useful DNA fragments in a certain environment can be suggested. For instance, the majority of colonies grow on pE plates rather than the pC and pCE when the transformation media includes Eryt as an inducer. This is clear evidence that the bacteria in BE have mostly uptaken the E fragment during the transformation. Additionally, the colony number on the pCE-driven from BCE culture stimulated with both Eryt and Chl together has significantly increased in comparison to BK, BC and BE. These are important outputs to assess decently whether there is a preference priority during the NT process since the bacteria must uptake both fragments E and C to grow up on the pCE. The data significantly overlaps with the idea claiming that the bacteria have a preference priority to uptake free DNAs during the NT.

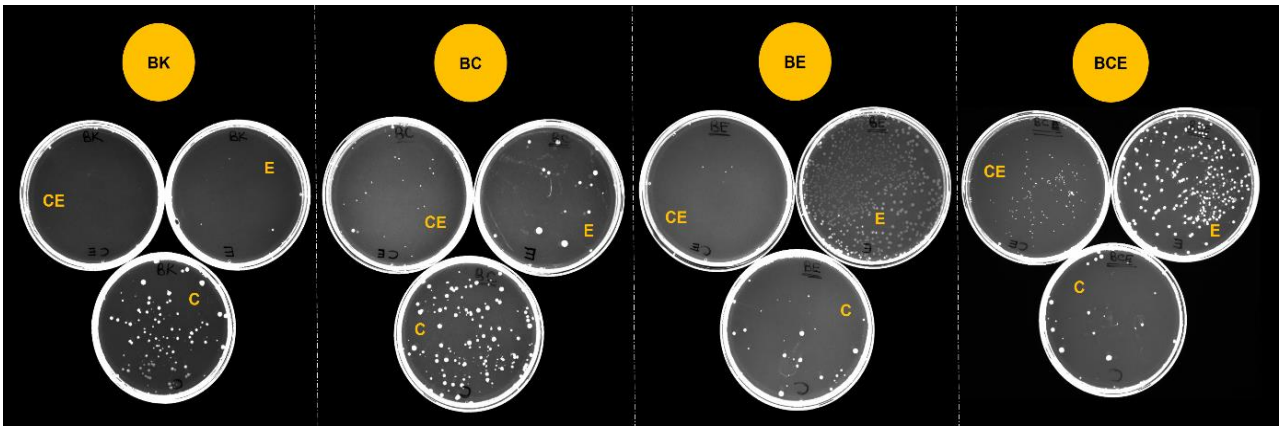

Fig. 2. Colony photographs on the selective agar media (pE, pC and PCE) for transformation cultures (BK, BC, BE and BCE). All transformation cultures basically include MNGE transformation medium with free DNA options (linearized C and E plasmids), and additionally have stress inducers. BK; control environment, no stress inducer. BC; including 50 ng Chl as a source of stress reminder. BE; containing 10 ng Eryt as a stress trigger. BCE; harbouring both 50 ng Chl and 10 ng Eryt as stress inducers. On the other hand, all selective media is LB agar including lethal antibiotics for wild-type B.subtilis. pBE; LB agar containing 1 µg/ml Eryt pBC; LB agar including 5 µg/ml Chl; pBCE; LB agar containing both 1 µg/ml Eryt and 5 µg/ml Chl.

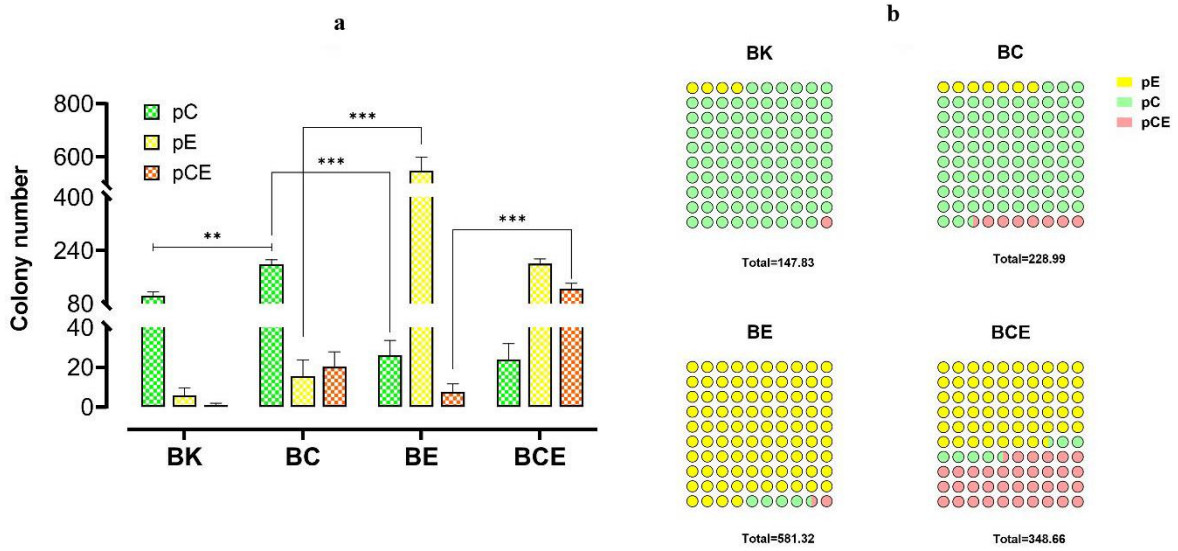

Fig. 3. Colony analysis for BK, BC, BE and BCE on pE, PC and PCE. **a.** The number of colonies on each plate has been counted and the data has been plotted in the grouped column graph for each culture on the specific selective media. pC is coloured greenish, pE is yellowish and pCE coloured redish. The data were shown as mean ± SEM ($n \geq 3$). $^{**}p<0.01$ BK vs BC, $^{***}p<0.01$ BC vs BE, **b.** the visualization of colony analysis with the spot graph. The colonies that appeared on each selective medium has been shown with different colors (pC-green, pE-yellow, pCE red). The number and distribution of colonies on selective media for each transformation medium have been displayed on an apart square. The spot graph has been plotted from the data of repeated samples ($n \geq 3$).

Previous studies revealed that the DNA transferred with the NT process either uses different species-specific uptake sequences or is sequence-independent (Nielsen & Elsas 2019). In the present study, to avoid structural and sequential differences, we used two equal-length DNA fragments (E and C), each with specific homologous sequences mediating to incorporate of a resistance gene into the *B. subtilis* genome (Härtl *et al*. 2001). According to the results, stimulating the bacteria with a definite stress factor during the NT process can lead them to uptake a certain DNA fragment which provides an adaptation advantage for stressful conditions. Therefore, we can suggest that the preference priority between E and C fragments during the NT process is decided with regard to the basic survival requirements of the bacteria (Moradigaravand & Engelstädter 2014, Di Giacomo *et al.* 2022. It is seen that Eryt has a stronger inducer impact on the bacteria compared to Chl when it comes to stimulating the bacteria for uptaking a certain DNA fragment. For instance, the majority of colonies from BC culture involving only Chl have formed on the pC, while the transformants from BCE and BE cultures including Eryt largely have grown on the pE or pCE instead of the pC (Figs 2, 3). Both Eryt and also Chl are potent antibiotics preventing bacterial translation elongation via binding ribosomal 50S region (Lin *et al*. 2005). Even though they compete for the same binding site on the 50S domain, Eryt has a stronger affinity to bind the region compared to Chl

(Taubman *et al*. 1966, Takada *et al.* 2022). This might explain why the cultures including Eryt have mostly directed the bacteria to fragment E rather than C during the NT process.

The similar pattern in the outputs of BK and BC is another conspicuous result that needs to be explained. BK is a control culture without including any inducer, but the majority of colonies based on BK culture have fragment C rather than other options like BC culture. In theory, the bacteria in BK culture should have uptaken DNA randomly among the options instead of having the preference priority. Although it was expected to see a random distributional colony growth on the pC and pE plates for BK culture, it has a similar output pattern to BC culture (Figs 2, 3b). Actually, the presence of a couple of colonies on pE and pCE for BC culture is coherent with all data, but almost all colonies from BK culture were seen only on pC. It can be speculated that the fragment C is preferable over fragment E by the bacteria due to the suitability of homologous sequences (Bordelet & Dubrana 2018). Although this preference might be associated with homologous sequences that fragment C has, frankly this phenomenon is hard to explain without further investigation.

Conclusion

The research question is to investigate whether microorganisms are capable of NT tends to select a particular free DNA over other options under certain environmental conditions. For this purpose, *B. subtilis* bacteria were tested in four different transformation media with two free DNA options. Transformation media were supplemented with inducers associated with free DNAs to understand the bacterial response to selection prompts. Cells from each culture medium were seeded on three different selective media to determine which free DNA the bacteria took up with NT. The research question was evaluated according to the analysis results of the colonies formed on the selective plates.

According to the obtained data, the stress trigger added to the transformation medium mostly led the bacteria to take up the DNA fragment associated with the stress source. Two remarkable results were obtained in the study. Although the number of colonies receiving E and C fragments is quite high in the presence of both triggers, the number of colonies that prefer E to C is also quite high. This can be explained by the fact that Eryt is an antibiotic stronger than Chl. Therefore, the bacteria may have selected fragment E more than C to avoid the effect of Eryt. Second, BK and BC cultures have similar output patterns on selective media. While the BK culture is expected to form random colonies on all selective media, the majority of colonies formed are on the pC plate. If the bacteria mostly uptake a particular DNA fragment even in the control environment, this in itself may indicate that the bacteria exhibit a preference priority in choosing free DNA. There can be several reasons behind this preference priority: for example, homologous recombination of the C fragment into the *B. subtilis* genome may be more favourable than in E (Bordelet & Dubrana 2018); C might have been preferred to E because of the obvious benefit of *lacA* gene in comparison to *amyE* in the BK culture condition *(Härtl et al.* 2001); *B. subtilis* might have an evolutionarily interest to fragment C rather than E (Liu *et al*. 2018, Yang *et al*. 2022). Future studies will help us to explain why a large percentage of bacteria in the BK medium prefer only the C fragment over other DNA options when there are no triggers in the medium.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: The authors confirm that the data supporting the findings of this study are available within the supplementary material of the article.

Conflict of Interest: The author has no conflicts of interest to declare.

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