# *Intra-species DNA exchange: Bacillus subtilis prefers sex* with less related strains

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*B. subtilis* is a soil dwelling bacterium with a diverse social life that includes quorum sensing-regulated interactions [1]. These interactions result in bacterial equivalent of sex, and subsequently have a profound influence on bacterial evolution[2]. Sexual isolation in *B. subtilis* predicts for more frequent uptake of DNA isolated from closely related strains[3-5], but DNA exchange between two interacting *B. subtilis* strains has never been addressed previously. Recently we discovered kin discrimination among highly related strains of *B. subtilis*, where less related strains showed antagonistic behaviour towards each other in the form of killing[6]. Here we show that antagonistic interactions between two less closely related *B. subtilis* strains result in increased recombination, which is in contrast to current dogma. We demonstrate that the induction of competence between non-kin strains is responsible for the observed elevated DNA uptake, which, through increased genetic variation, can increase the rate of adaptation as demonstrated here by the successful exploitation of a novel adaptive zone by a recombinant strain. Our results demonstrate an important evolutionary mechanism of ''promiscuous but safe sex'': a type of bacterial cell-contact dependent DNA exchange that could promote diversification of conspecifics and exclude non-specific and potentially risky DNA of other species. Our findings could help understand the vast diversity of *B. subtilis* species at the genomic level despite existing mechanisms limiting less-related DNA integration during transformation. It is possible that this (or similar) mechanism could be accountable for the diversification of many other bacterial species capable of natural transformation.

Sexuality in bacteria has evolved and persisted through evolution because it allows bacteria to obtain novel genetic combinations and promotes adaptation [7]. Natural genetic transformation allows bacteria to take up extracellular DNA and integrate it into their genome. It is the only horizontal gene transfer (HGT) mechanism (the other major two being conjugation and transduction) that is entirely encoded on the chromosome and therefore could have evolved as a form of "bacterial sex"[8,9]. This topic remains controversial as it is still uncertain whether bacterial sex evolved to maximize diversity and adaptation, to preserve/repair the existing genes [10,11], or if it serves as a means to uptake and use DNA for food[12]. Most of the studies on bacterial sex focus on the consequences of HGT, such as gene rearrangements, adaptation, fitness of offspring, etc, however, little is known about DNA exchange in mixed bacterial populations, the DNA origin preference or how bacterial interactions affect DNA transfer between conspecifics[13,14].

The model gram positive bacterium *Bacillus subtilis* is naturally competent [15] but transformation has almost exclusively been studied in liquid cultures using laboratory strains and exogenously added DNA. Experiments have shown that transformation frequency decreased dramatically with donor/recipient sequence divergence indicating more frequent uptake of DNA isolated from closely related microorganisms (sexual isolation) [3-5], however, DNA exchange in multicellular communities has not been addressed before.

*B. subtilis* has been shown to form boundaries between distinct swarms through kin discrimination (KD). Swarm boundaries between less closely related strains (< 99,4% nucleotide identity at four housekeeping loci) are highly distinct, which defines them as non-kin, whereas highly related strain pairs (above 99,9% nucleotide identity) display swarms merging as the predominant phenotype and are therefore considered kin [6]. KD genes encode for a rich arsenal of regulators, cell-surface modifiers, and intercellular attack and defence

molecules, generally antimicrobials [16]. Why *B. subtilis* has radiated into many different KD types and how this diversity is maintained is not well-understood nor do we understand how KD influences horizontal gene transfer.

First, we obtained the whole genome sequences of the six wild type *B. subtilis* strains and calculated their degree of relatedness using average nucleotide identity (Supplementary Table 1) [17]. This confirmed that strains, which show merging phenotypes, were highly related (99.94-99.97% ANI), whereas boundary forming strains were less closely related (98.63-98.74 %) (Supplementary Table 1). Next, we tested whether transformation-mediated gene transfer occurs more frequently in swarm pairs that form boundaries, compared to swarm pairs that merge. Differentially marked (spectinomycin ($Sp^R$) and chloramphenicol ($Cm^R$)) pairs of kin strains (merging phenotype), non-kin strains (boundary phenotype) and differentially marked isogenic strains (control 'self-merging' phenotype) were inoculated on agar (Fig. 1a). DNA exchange frequency, calculated as the proportion of double marker mutants isolated from the swarm boundary divided by the total colony count was significantly higher for non-kin strain pairs compared to kin strain pairs or the isogenic controls (2 tailed t-test; $p<0.05$, see Supplementary Information) (Fig. 1b, Supplementary Fig. 1a). Experiments using strains with inverse marker combinations yielded statistically similar results (Supplementary Fig. 1b, see Supplementary Information). Mixing of differentially marked strains (1:1) in shaken co-cultures did not result in increased non-kin DNA exchange (Supplementary Fig. 1c, d) indicating the importance of cell-cell contact in DNA transfer.

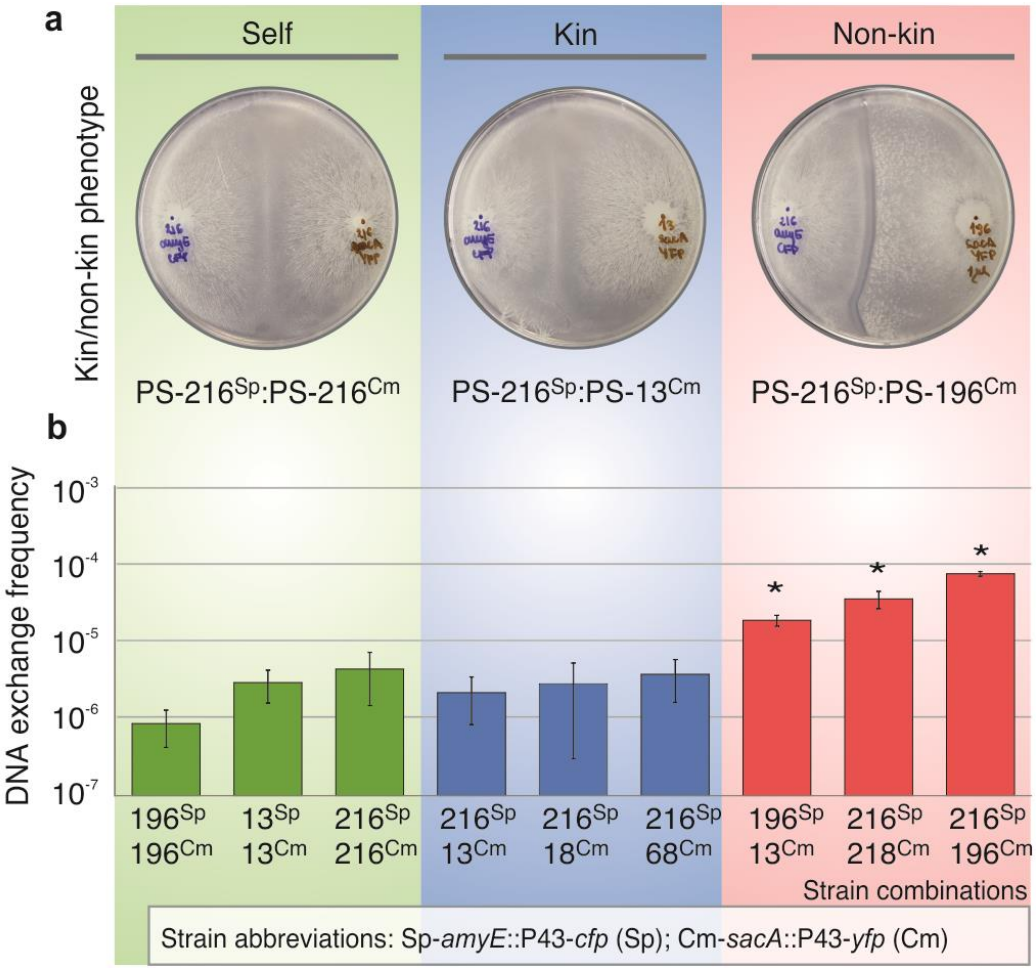


**Fig. 1. DNA exchange frequency. a**, Kin discrimination phenotypes. Self-interaction (green), kin interaction (blue), and non-kin interaction (red). **b**, DNA exchange frequency. Self, kin and non-kin strain pairs are depicted by green, blue and red bars, respectively. Strain abbreviations are as follows PS-216$^{Sp}$ (*amyE*::P43-*cfp* ($Sp^R$) and PS-216$^{Cm}$ (*sacA*::P43-*yfp* ($Cm^R$). This holds for all strains (e.g. PS-216 the same as 216). All experiments were performed in three independent experiments using three replicates. Error bars represent SD of the average values. * represent statistically significant values (two tailed t-test, see Supplementary Information for details).

Areas where kin strains merge contained intact cells, whereas swarm boundaries between non-kin strains showed deflated and empty cells pointing to the cell lysis (Fig. 2). Lysis of neighbouring cells followed by the successful uptake of genomic DNA thus released has been demonstrated in *Vibrio cholerae*[13], and *Streptococcus pneumoniae* [18,19]. Extracellular DNA is also a prerequisite for *B. subtilis* swarm DNA exchange (Supplementary Fig. 2), however, DNA concentrations measured at the boundary were comparable to those between kin strains (Supplementary Fig. 3). The addition of exogenous DNA (Supplementary Fig. 4-5) and inactivation of DNAses genes in interacting strains (Supplementary Fig. 5a,b, see Supplementary Information) did not affect DNA exchange (two tailed t-test, $p$=0,1062) indicating that the uptake and recombination of free DNA between non-kin is not limited by DNA availability (Supplementary Fig. 5a, see Supplementary Information).

The above experiments on DNA exchange were based on the detection of double mutants, which in theory could have arisen via bidirectional or unidirectional DNA uptake and recombination. To distinguish between these scenarios, we constructed *comGA* mutants with impaired DNA uptake efficiency [20] and paired a wild type strain with a non-kin *comGA* mutant strain (WT PS-216 with PS-196 Δ*comGA* and PS-196 with PS-216 Δ*comGA*). PS-216 increases DNA marker uptake ~7-fold when inoculated opposite PS-196 relative to the isogenic control ($p$=0.0002), whereas PS-196 displays decreased DNA marker uptake upon encounter with PS-216, however not significantly (Fig. 2b) ($p$=0.21). DNA uptake was not detectable when pairing both Δ*comGA* mutants (data not shown). This experiment shows that DNA uptake and recombination primarily take place in one strain. Observation of damaged cells at the boundary motivated us to test if the less-competent strain is predominantly attacked and lysed. We compared the relative fitness (strain frequency) of both strains at the boundary to the fitness of each strain paired with an isogenic marked mutant. PS-216 exhibits a significant fitness increase ($p$=0.01) whereas PS-196 exhibits a dramatic reduction in fitness at the swarm boundary ($p$=0.01), suggesting the lysis of PS-196 by PS-216 strain (Fig. 2c).

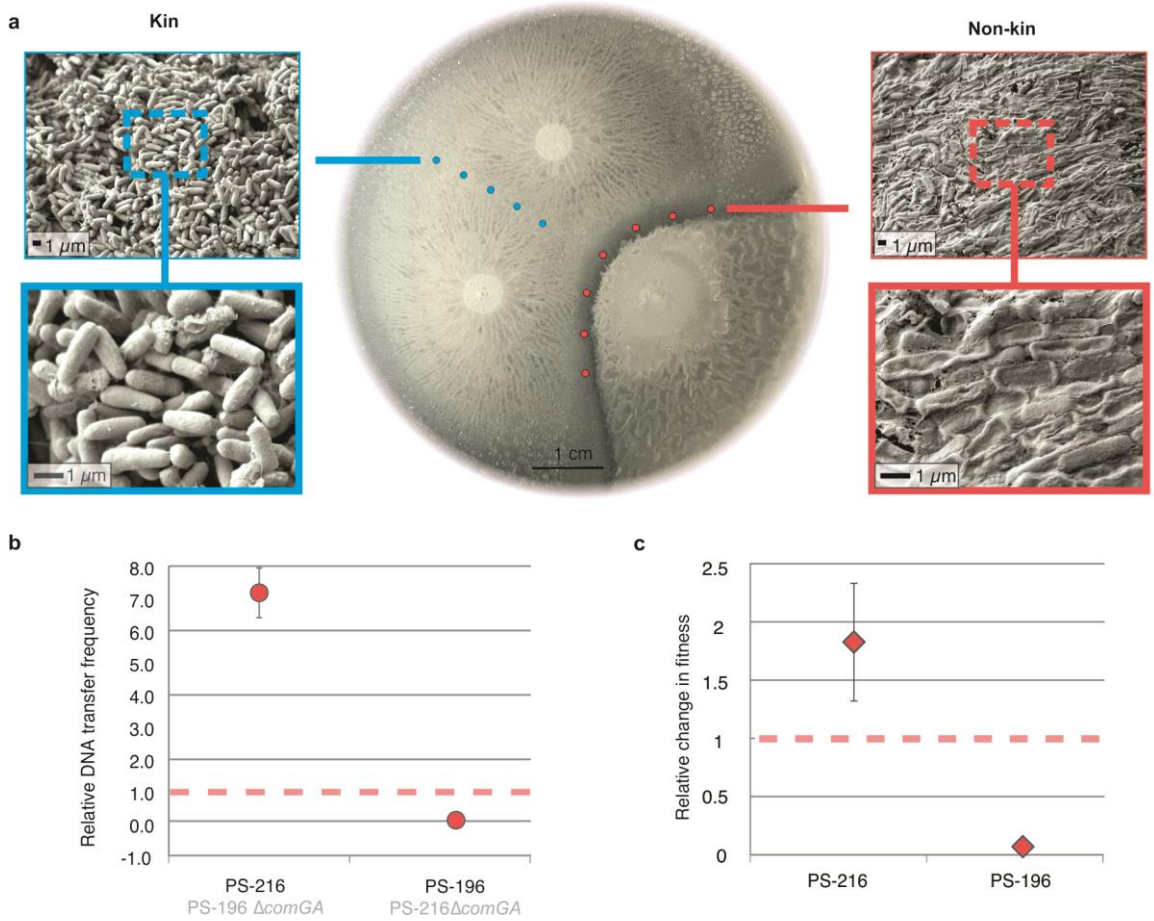


**Fig. 2. The effect of KD on cell integrity, DNA exchange and fitness. a,** Swarm assay on B agar. The left and right panels are scanning electron microscopy micrographs taken at the meeting point of kin strains (blue, left) PS-216:PS-13 and non-kin boundary (red, right) PS-216:PS-196. **b,** Relative DNA transfer frequency of PS-216 (left) and PS-196 (right) at the boundary when paired with a non-competent non-kin strain carrying inactivated *comGA* (indicated with grey letters) versus the isogenic strain. **c,** Relative change in strain frequency of PS-216 and PS-196 at the boundary when paired with non-kin as opposed to isogenic strain. All experiments were performed in three independent experiments in three replicates. Error bars represent SD of the average values. Strain genotypes: PS-216 (*amyE*::P43-*cfp*, $Sp^R$), PS-13 (*sacA*::P43-*yfp*, $Cm^R$), PS-196 (*sacA*::P43-*yfp*,$Cm^R$).

Stress and the release of antimicrobials have been shown to induce competence in many species[11,21-23]. The *sigW* ($\sigma^W$) gene is expressed in response to cell envelope stress [24] and was previously shown to be up-regulated at the swarm boundary [16]. To examine the connection between stress and competence we measured the expression of P*sigW-yfp* ($\sigma^W$) and DNA exchange in Δ*sigW* (σW) mutants in both the swarm boundary and merging area. As expression of $\sigma^W$ was more prominent at the boundary (Fig. 3a) we hypothesized that an inability to respond to stress (Δ*sigW*) in non-kin encounters would prevent the induction of competence. Indeed, DNA exchange between non-kin Δ*sigW* strains (PS-216 and PS-196) decreased significantly compared to wt non-kin strains ($p$=0.041 and $p$=0.005, respectively), while no changes occurred to the rate of DNA transfer in isogenic combinations (Fig. 3b, see Supplementary Information).

To test whether antagonistic interactions result in up-regulated competence at the boundary, the central competence *comGA* gene in strain PS-216 was tagged with the *yfp* reporter. This tagged strain was then paired with three different strains: i) a wt version of itself (PS-216), ii) a wt kin strain (PS-13) and iii) a wt non-kin strain (PS-196) (Fig. 3c,d). Significant up-regulation of *comGA* was observed at the boundary of non-kin strains, compared to *comGA-yfp* activity of self ($p$=0.030) and kin ($p$=0.0287), and no difference was observed between kin and self *comGA-yfp* activation ($p$=0.598) (Fig. 3c,d). These results show that competence is induced at the meeting point of less related non-kin strains and furthermore that competence is enhanced via cell-envelope stress response.

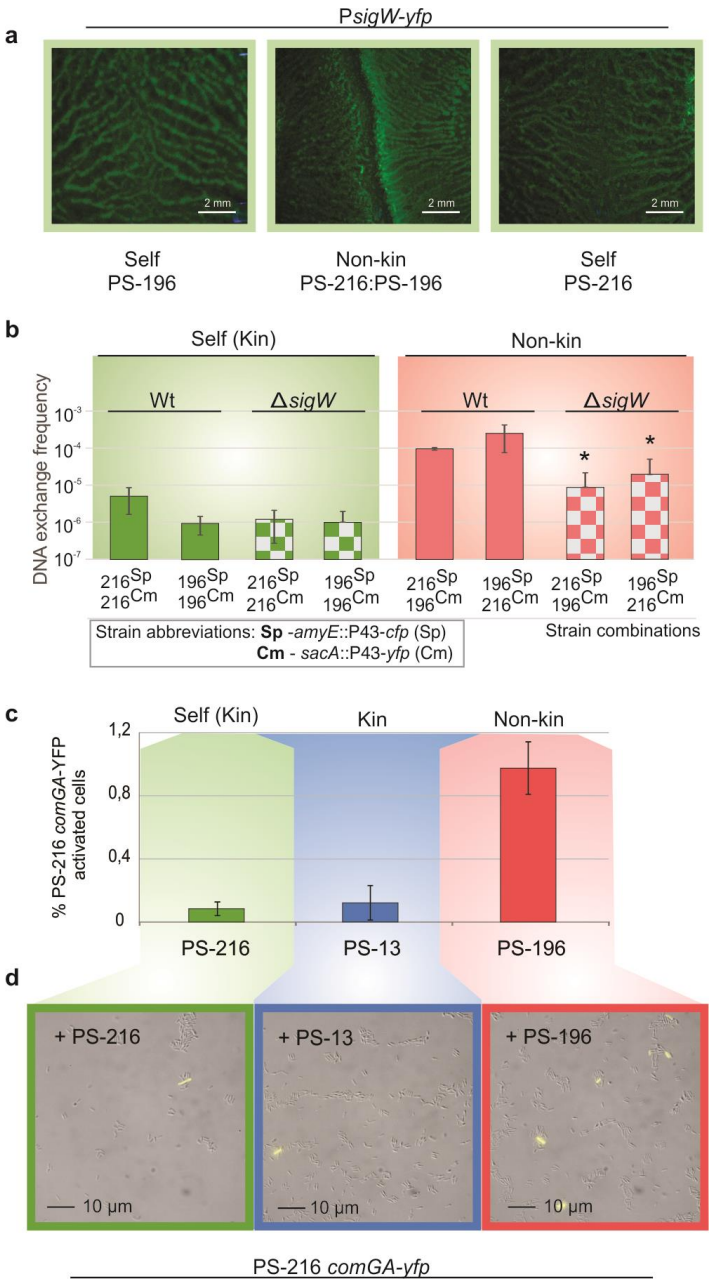


**Fig. 3. Stress and competence induction. a,** Meeting points of self and non-kin *sigW-yfp* swarms. **b,** The effect of stress response inactivation (Δ*sigW*) on DNA exchange. Green depicts self-combinations and red depicts non-kin combinations. Full columns and chequered columns represent DNA exchange between wt controls and Δ*sigW* mutants, respectively. **c,** Activation of competence genes in strain PS-216 *comGA-yfp* at the meeting point of self (PS-216$^{Sp}$), kin (PS-13$^{Sp}$) and non-kin (PS-196$^{Sp}$) strain. Bars represent fractions of PS-216 cells expressing the *comGA* gene at swarm meeting points. Error bars represent SD of the average values of at least three replicates from three independent experiments. **d,** Overlays of transmitted and fluorescent light micrographs of PS-216 cells expressing *comGA-yfp* are shown in the same order as above. Shown are representative single images from one experiment of at least three replicates from three independent experiments. * represent statistically significant values (two tailed t-test, see Supplementary Information for details).

As the uptake and recombination of extracellular DNA is an integral part of transformation, it is often assumed that recombination-based benefits are at least partly responsible for its evolutionary maintenance [7]. There is evidence across model systems that transformation can result in the uptake of alleles or genes with a beneficial effect on fitness [25]. To test whether KD-mediated transformation can aid adaptation through the uptake of beneficial alleles originating from a non-kin swarm, a swarm plate containing one half of B medium and other half B medium supplemented with Sp+Cm (Fig. 4a) was inoculated with Cm-marked PS-196 (p43-*yfp*) and Sp-marked PS-216 (p43-*cfp*) (Fig. 4a). PS-216 invaded the Sp+Cm supplemented half of the plate from the boundary (Fig. 4b), allowing the parent PS-216 strain to spread with the transformants, however, limiting invasion of ancestral donor PS-196 (Fig. 4c).

Transformed PS-216, carrying both antibiotics and isolated from the invading area, also carried both fluorescent markers (*cfp* and *yfp*) (Fig. 4d) confirming that the invading lineage was the result of a transformation event. No spontaneous mutants were observed when wt PS-216 or PS-196 strains were tested (Supplementary Fig. 6). This result demonstrates that KD-mediated transformation can enable adaptation to novel selective pressures.

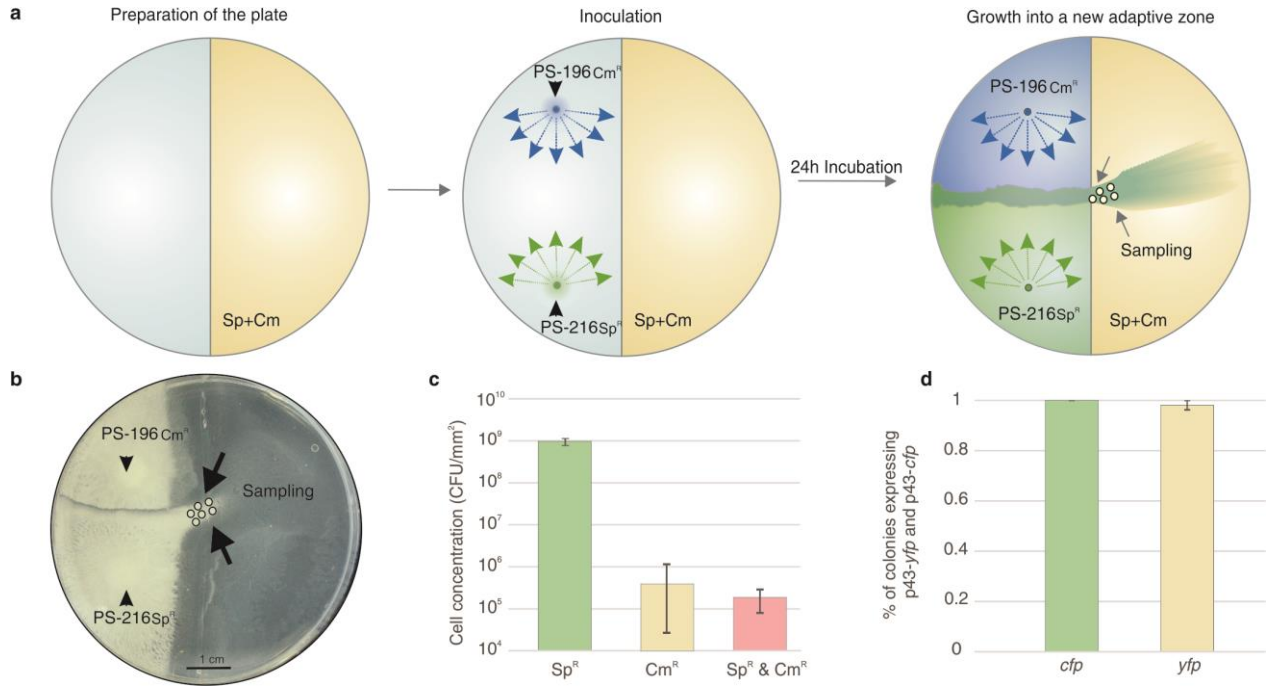


**Fig. 4**. **DNA provides ecological advantage. a,** Experimental scheme. Samples were taken from the area that spread onto the part of plate containing both antibiotics (marked with arrows). **b,** DNA exchange advantage assay between strains PS-216 (*amyE*::P43-*cfp*, $Sp^R$) and PS-196 (*sacA*::P43-*yfp*, $Cm^R$). PS-216 was able to colonize the dual antibiotic area (indicated by black arrows) from which samples (depicted with circles and arrows) were taken. **c,** CFU of PS-216, PS-196 and transformants at the sampling points. **d,** The ratio of colonies expressing both *cfp* and *yfp* fluorescent proteins, isolated from the dual Ab sampling area. All experiments were performed in three independent experiments. Error bars represent SD of the average values.

Competence development is induced by pherotype-specific pheromones and strains carrying different pheromones have been shown to inhibit each other's competence development in liquid media [26-28]. This is the first study showing competence up-regulation resulting from antagonistic interactions between different pherotypes and consequently higher inter-pherotype DNA exchange. The efficiency of transformation-mediated recombination of more divergent DNA fragments has been shown to decrease in a log-linear fashion in *Bacillus* [4,29]. However, our results counteract the view of 'sexual isolation'; although recombination between more divergent DNA itself is less efficient, this is/must be outweighed by more efficient DNA uptake following KD-mediated competence induction.

Together, our findings suggest that intra-species bacterial sex is encouraged between less closely related (non-kin) conspecifics through a kin-discrimination dependent competence up-regulation mechanism, linked to *sigW*-mediated stress response. By coupling competence with lysis of related neighbouring strains, the probability of efficient incorporation of novel alleles and genes that have proved to function in a genomically and ecologically similar context is maximised. Our findings support the DNA uptake for "genome diversification" hypothesis instead of "genome repair" or "DNA for food" hypothesis amongst closely related *Bacillus subtilis* strains [10-12] Interestingly, the antagonism observed between less related conspecifics has been shown to extend to closely related species, but break down when more divergent species are encountered [30], possibly preventing competence induction between strains that are too divergent.

It could be said that *B. subtilis* practices "promiscuous but safe sex", a type of DNA exchange between divergent strains, which promotes genetic diversification that underpins adaptation.

## Methods

**Strains and media.** Strains used in this study and their mutant derivatives are presented in Supplementary Table 2. Strain combinations used in experiments are shown in Supplementary Table 3. Briefly, 6 *Bacillus subtilis* wild type strains isolated from sandy bank of the Sava River in Slovenia [28] were used**.** For strains carrying antibiotic markers appropriate antibiotics at the following concentrations were used: 5 µg/ml of chloramphenicol (Cm), 100 µg/ml of spectinomycin (Sp), 20 µg/ml of tetracycline (Tet), 25 µg/ml of kanamycin (Kn) and 20 µg/ml of erythromycin (Ery). Overnight cultures were prepared in liquid LB medium and swarming assays were performed on swarming agar (final agar concentration 0.7%), which was based on B-medium composed of 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4 \times 7H_2O$, 27 mM KCl, 7 mM sodium citrate×$2H_2O$, 50 mM Tris-HCl, pH 7.5; 2 mM $CaCl_2 \times 2H_2O$, 1 µM $FeSO_4 \times 7H_2O$, 10 µM $MnSO_4 \times 4H_2O$, 0.6 mM $KH_2PO_4$, 4.5 mM sodium glutamate, 0.86 mM lysine, 0.78 mM tryptophan and 0.2% glucose.

**Strain construction.** For fluorescence visualization experiments wild type *B. subtilis* strains were tagged with a *yfp* or a *cfp* gene linked to a constitutive promoter (p43), inserted at the *sacA* or *amyE* locus, and for observation of competence induction a *yfp* gene was linked to the *comGA* promoter. P43-*yfp* construct from Pkm3-p43-*yfp* plasmid [6] was digested with EcorI and BamHI and ligated into the ECE174 [31] plasmid yielding the pEM1071 plasmid carrying a p43-*yfp* fusion inside the *sacA* integration site. For construction of p43-*cfp* fusion PkM8 plasmid was used, carrying the *cfp* gene linked to *spoIIQ* region [32]. *spoIIQ* region from the original plasmid was removed by EcoRI and HindIII digestion and replaced by p43 promoter sequence, which was amplified with primers p43-F1-EcoRI and p43-R1-HindIII [6] and was ligated into the PkM8 EcoRI and HindIII sites yielding the Pkm8-p43-*cfp* pEM1069 plasmid.

Strains carrying P*comGA-yfp* (Cm) and knockouts in Δ*comK* (Kn), Δ*nucB* (kn), Δ*yhcR* (Ery), Δ*comGA* (Ery), Δ*epsA-O* (Tet) and Δ*comQXP* (Kn), *sigW-yfp* (Ery) and Δ*sigW* were obtained by transforming the wt *B. subtilis* strains with DNA isolated from *B. subtilis* 11A79 (*PcomGA-yfp*) [33], BD2121 (Δ*comK)* [34], BKK25750 (Δ*nucB*) [35], BKE09190 (Δ*yhcR*) [35], BKK24730 (Δ*comGA*, kn) [35], BKE24730 (Δ*comGA*) [35], ZK4300 (Δ*epsA-O*) [16], plasmid pED302 (Δcom*QXP*) [27], NL362 (Δ*sigW*) [16] and ZK4860 (*sigW-yfp*) [16] (Supplementary Table 2). The DNA (plasmid or genomic) was introduced to the *B. subtilis* using standard transformation protocol.

**Harvesting spores.** For swarming assay spores were inoculated on semi solid media and spore crops were prepared by inoculating an overnight *B. subtilis* culture from LB medium to 2 x SG sporulation medium [36]. For detailed protocol see Supplemental Information.

**Swarm boundary assay.** Swarming assays were performed as previously described [6], with a few minor adaptations. *B. subtilis* spores were diluted 1:1 with LB media and 1-2 µl was inoculated in parallel on semisolid 0.7% agar B media. Pairs of inoculated kin or non-kin strains were labelled with different antibiotic resistance and fluorescence marker. When spores were unavailable, overnight cultures, prepared from the freezing stocks (-80°) were transferred to fresh liquid LB media (1% inoculum) and incubated for 2 h with shaking (200 rpm) at 37°C. After 2 h incubation, the culture was again transferred to fresh liquid LB media (1% inoculum) and incubated with shaking (200 rpm) at 37°C for additional 2 hours. After the second incubation the exponential phase cell suspension was used for B media inoculation (for experimental scheme see Supplementary Fig. 4a, samples were taken in the same manner for CFU or DNA quantification). Inoculated agar plates were incubated overnight (22-24 h) at 37°C and 80 % humidity. If not stated otherwise, experiments were performed in three biological replicates and each replicate was sampled in three technical replicates.

**Frequency of DNA exchange between swarms of *B. subtilis* strains on semisolid B media.** To determine the frequency of DNA exchange between kin or non-kin *B. subtilis* swarms, pairs of strains were inoculated parallel to each other on semisolid B medium and incubated overnight as described above. Ten samples (per plate) were taken at the swarm meeting point with cut pipette tips and resuspended in 500 µl 0.9% NaCl solution. Using appropriate antibiotic selection, CFUs of each approaching swarms at the meeting point was determined as well as the number of cells that have taken up each other's DNA. DNA exchange frequency at the border was calculated as the number of total CFU/ml of transformants (Cm+Sp) divided by total CFU count (sum of CFUs of both strains (Cm and Sp) minus the number of transformants (Cm+Sp)).

**DNA exchange in the presence/absence of DNaseI.** In order to quantify DNA exchange in the presence of DNaseI, 20 µl of DNaseI (5 mg/ml) was spread on one half of the semisolid B media (concentration on the surface of media was 50 µg/ml) (Supplementary Fig. 2). *B. subtilis* strains were inoculated parallel to each other and incubated overnight for swarms to spread as described above. Sampling was performed at the swarm meeting point of non-kin strains in the area containing DNaseI and in the area without DNaseI present (Supplementary Fig. 2). Ten samples were picked from each area (with/without DNaseI) using cut pipette tips and resuspended in 500 µl 0.9% NaCl solution. CFUs were determined and DNA exchange was calculated as described above.

***comGA* gene expression.** The *comGA-yfp* gene activation was observed at meeting points of kin and non-kin swarms. Pairs of kin and non-kin strains were inoculated parallel to each other on semisolid B media and incubated overnight as described above. Samples were taken at the meeting points of two swarms with an inoculation loop, resuspended in 200 µl of 0.9% NaCl solution and mixed with a vortex mixer. 20 µl of each sample was pipetted onto a glass slide coated with a solution of poly-L-lysine (0.1% (w/v). Before microscopic observation, slides were dried and antifade reagent SlowFade (Invitrogen, ZDA) was added. Zeiss Axio Observer Z1 with DIC technique (differential interference contrast) and fluorescence technique with fluorescence filter (Zeiss, Göttingen, Nemčija) was used for observing fluorescent protein *yfp* (Ex 430 nm/Em 474 nm). For every technical

replicate 20 fields of view were analysed and photographed totalling to approximately 30.000 cells per studied interaction (self, kin or non-kin). Pictures were analysed with Fiji (Life-Line version). Overall number of cells (DIC technique) and number of cells expressing *comGA-yfp* (yellow glowing cells under microscope with fluorescence filter for observing *yfp*) were determined. The ratio of cells expressing *comGA-yfp* was calculated.

**DNA quantification.** DNA concentration was determined in individual *B. subtilis* swarms and at swarm meeting points of kin or non-kin strains. Pairs of strains were inoculated parallel to each other on semisolid B media and incubated overnight as described above. Twenty samples were taken at the swarm meeting points and within each swarm with cut pipette tips (Supplementary Fig. 3). Samples were first resuspended in 800 µl Gel Solubilization Buffer and lightly mixed. DNA was isolated using PureLink® Quick Gel Extraction Kit (Thermo Scientific, ZDA). Samples were heated for 20 min at 50°C fot the agar to melt. Further DNA isolation process was performed as specified in PureLink® Quick Gel Extraction Kit manual. DNA was eluted with 330 µl 1x TE buffer and 100 µl of DNA samples were pipetted into a 96-well microtiter plate where DNA concentration was determined using QuantiFluor® dsDNA System (Promega Corporation, ZDA) according to the manual. The DNA concentration in the agar was calculated using calibration curve obtained from DNA isolation of Lambda DNA standard (QuantiFluor® dsDNA System) added to the agar plate in concentrations: 0 pg/µl, 10 pg/µl, 50 pg/µl and 100 pg/µl.

**DNA quantification and DNA exchange in the presence of exogenous DNA.** To test whether DNA at the meeting point gets digested by *B. subtilis* nucleases exported to the media and whether or not this affects the DNA exchange frequency we simultaneously measured DNA concentration and DNA exchange in plates with/without added DNA at the centre of the plate (Supplementary Fig. 4) where PS-216 (*amy*:p43-*cfp* (Sp) and PS-196 (*sacA*:p43-*yfp* (Cm) swarms met. Approximately 30 µg of DNA (PS-216 Δ*epsA-O* (Tet)) was added to a 1 cm wide stripe in the middle of the B medium agar plates, where the strains usually meet and 20 samples from the meeting point on B medium plate with/without added DNA were resuspended in 700 µl of 0.9 % NaCl instead of Gel Solubilization Buffer from the PureLink® Quick Gel Extraction Kit (Thermo Scientific, ZDA) to prevent damaging viable cells. 100uL was used to determine DNA exchange frequency (described above) and the remaining sample was centrifuged at 10000 g for 5 min and filtered through a 0.2 µm pore Millipore filter (Merck, KGaA, Germany) previously wetted with 1 ml of 0.9% NaCl solution. DNA concentration was determined using QuantiFluor® dsDNA System as described above.

**DNA quantification and DNA exchange between nuclease mutant strains in the presence of exogenous DNA.** To test whether more DNA is found in the agar if nuclease mutant strains are used, and how this affects DNA exchange of the nuclease mutant strains, we simultaneously measured DNA concentration and DNA exchange at the boundary of PS-216 (*amy*:p43-*cfp* (Sp), Δ*nucB* (Kn) Δ*yhcR* (Ery)) and PS-196 (*sacA*:p43-*yfp* (Cm), Δ*yhcR* (Ery)) with/without added DNA at the centre of the plate. DNA quantification and the following procedures are the same as decribed above for wild type strains.

**Double plate setup for investigating ecological advantage of transformants.** The spread of transformants, cells which acquired the genes from the opposite swarm (*amyE*::p43-*cfp* or *sacA*::p43-*yfp)* on the boundary, that now carry both markers, into area containing two antibiotic markers was tested on B medium agar plate. One half contained semi solid B medium (0.7% agar) and the other half contained semi solid B medium supplemented with two antibiotics: 10 µg/ml of Chloramphenicol (Cm) and 100 µg/ml of Spectinomycin (Sp) (Fig. 4). First, B media without antibiotics was poured and allowed to solidify, after which one half was carefully removed with a sterile scalpel and B media containing both antibiotics was poured into the plate. Strains (kin and non-kin pairs) were inoculated onto the agar containing no antibiotics and the spread of transformants was observed and sampled after 24h of incubation at 37°C at 80% humidity (Fig. 4). The number of transformants colonizing the area containing antibiotics and total CFU was determined by selective plating. 50 colonies carrying both Sp and Cm from three independent experiments were further tested for *yfp* and *cfp* fluorescence to confirm the transfer of DNA.

**DNA exchange advantage assay.** Pairs of non kin strains (PS-216 (*amyE*::P43-*cfp* (Sp) and PS-196 *sacA*::P43-*yfp* (Cm)) were tested on “DNA exchange advantage” swarm agar plates. One half of the plate consisted of B medium and two non-kin strains were allowed to swarm towards each other without the presence of antibiotics (Fig. 4). The other half of the plate was supplemented with two antibiotics (Sp and Cm) and spreading into the region containing both antibiotics was only allowed for DNA exchange transformants, carrying both antibiotic resistance genes (Sp and Cm) (Fig. 4). The observed spreading area was sampled (Fig 4b) and inoculated onto agar plates containing Sp, Cm and Sp +Cm. The majority of the population isolated from the spread area consisted of PS-216 (*amyE*::P43-*cfp* (Sp))(Fig. 4c), despite supplementation with two antibiotics, suggesting that the minority of transformants, carrying both Ab resistance genes helped the ancestor strain to spread by degrading antibiotics. Next, 50 randomly selected colonies obtained from Sp+Cm plates from 3 individual experiments ($n_{total}$=150), were tested for *yfp* and *cfp* fluorescence with 100 and 98 % of all tested colonies growing on Sp and Cm (n=150) were positive for *cfp* and *yfp* fluorescence, respectively (Fig. 4d). When wild type strains were used for the “DNA exchange advantage” assay no spreading was observed after 24 or 48 hours of incubation demonstrating that spreading into area containing two antibiotics is the consequence of active marker gene acquisition and not spontaneous mutations at the boundary (Supplementary Fig 6).

**Relative DNA exchange frequencies in non-kin vs. self.** In order to determine whether DNA exchange frequency of swarming *B. subtilis* PS-216 strain in non-kin combination is significantly higher compared to DNA exchange frequency with an isogenic strain, we used pairs of strains in which one of the strain carried a Δ*comGA* mutation and was not able to accept DNA. This enabled us to determine DNA exchange

frequency of only one strain in a pair (kin or non-kin pair) and not total DNA exchange frequency as described above, where transformants of both strains were summed in the equation. Pairs of strains were inoculated parallel to each other on semisolid B medium, incubated overnight and sampled as described above. DNA exchange frequency was calculated as the number of total CFU/ml of transformants (Sp+Cm) divided by CFU count of the strain not carrying the *comGA* mutation. Relative DNA exchange frequencies were calculated as DNA exchange frequency of a particular strain in non-kin combination divided by DNA exchange frequency of strain in combination with their isogenic strain (non-kin DNA exchange/self DNA exchange).

**Scanning electron microscopy.** For observation of the bacterial surface, the selected pieces of colonies were cut from agar plates and fixed in 1% glutaraldehyde and 0.5% formaldehyde in 0.1 M cacodylate buffer, pH 7.3 at 4°C overnight. After washing of the fixative with 0.1 M cacodylate buffer, the samples were postfixed in 1% aqueous solution of $OsO_4$ for 1 h. Postfixed samples were dehydrated in an ascending ethanol series (30, 50, 70, 90, and 96%) and transferred into pure acetone that was gradually replaced by hexamethyldisilazane (HMDS) and allowed to air-dry overnight. Dried samples were attached to metal holders with silver paint, coated with platinum and observed with a JEOL JSM-7500F field-emission scanning electron microscope.

**Fitness calculations.** The boundary between non-kin strains PS-216 (*amyE*::P43-*cfp* (Sp) and PS-196 *sacA*::P43-*yfp* (Cm) strains was sampled and CFU of each strain was determined in the boundary ($CFU_{216non\text{-}kin}$, $CFU_{196non\text{-}kin}$) and total CFU of both strains ($CFU_{non\text{-}kin(216+196)}$) was determined. Each strain was also staged with a self strain carrying a different Ab marker and CFU of each strain was determined ($CFU_{216kin}$, $CFU_{196kin}$), and total CFU was determined ($CFU_{kin(216(Sp)+216(Cm)}$, $CFU_{kin(196(Sp)+196(Cm))}$). Fitness was calculated as relative change in strain frequency in non-kin versus kin setting. Relative change in the frequency of strain PS-216 in non-kin setting versus kin setting is shown in the equation below.

Fitness (relative change in strain frequency in nonkin vs kin setting) =

$$(CFU_{(216non\text{-}kin)}/CFU_{(non\text{-}kin(216+196)})/(CFU_{(216kin)}/CFU_{kin(216(Sp)+216(Cm))})$$

Strain PS-216 exhibits a significant fitness increase in non-kin combination compared to kin (two tailed t-test, $p$=0.01) and PS-196 exhibits a dramatic reduction in fitness reduction at the swarm boundary, demonstrating PS-216 lyses PS-196 ($fitness_{216}$=1.82, $p_{216}$=0.01; $fitness_{196}$=0.07, $p_{196}$=0.01; two tailed t-test,) (Fig. 2c).

**Bioinformatics.** Raw reads for each isolate were obtained after sequencing of dual-indexed Nextera XT libraries (Illumina, USA) prepared from single isolates DNA extracts. Sequencing of the pooled libraries was performed using an Illumina MiSeq benchtop sequencer and 2x250bp paired-end reads MiSeq v2 kit-500 cycles reagents (Illumina, USA). Raw reads were trimmed of remnant sequencing adapters sequences and low-quality regions (Phred score < Q20) using CLC Genomics Workbench 9.5.4. (https://www.qiagenbioinformatics.com/). Assembly was performed on adapters and quality trimmed reads with CLC Genomics Workbench 9 assembler using default parameters. Genome sequences are available in the NCBI database under genome accession numbers VBRL00000000, VBRM00000000, VBRN00000000, VBRO00000000, VBRQ00000000 and VBRR00000000.

Genome analysis._The degree of relatedness between the 6 *B. subtilis* isolates was evaluated using Average Nucleotide Identity [37], using BLAST similarity scores of 1020bp long genome segments between all genome pairs (ANIb) as described by Goris et al. [38]. The ANI computation was ran using anvi'o v.5.4 [39] implementation of pyANI v 0.2.7 (http://widdowquinn.github.io/pyani/)[40]. Genome sequences are available in the NCBI database under genome accession numbers VBRL00000000, VBRM00000000, VBRN00000000, VBRO00000000, VBRQ00000000 and VBRR00000000.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request. Genome sequences are available in the NCBI database under genome accession numbers VBRL00000000, VBRM00000000, VBRN00000000, VBRO00000000, VBRQ00000000 and VBRR00000000.

**Code availability**

The ANI vas ran using anvi'o v.5.4 implementation of pyANI v 0.2.7 available at http://widdowquinn.github.io/pyani/.

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**Authors contributions**

P.S. and I.M.M conceived the project, P.S., K.B., and R.K., performed the experiments. P.S., J.K., J.N. and J.M. performed the genome sequencing and assembly analysis. P.S., K.B., R.K., J.N. and J.M. contributed with the methods and P.S. produced the figures. P.S. and I.M.M supervised the experiments. P.S., M.V. and I.M.M. wrote the manuscript, with all authors contributing to the final version.

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**Competing interests** The authors declare no competing interests.

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