Published in final edited form as:
Trends Microbiol. 2008 June ; 16(6): 269. doi:10.1016/j.tim.2008.03.004.

Ecology and genomics of *Bacillus subtilis*

Ashlee M. Earl[1], **Richard Losick**[2], and **Roberto Kolter**[1,*]

[1] Dept. of Microbiology & Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA

[2] Department of Molecular & Cellular Biology, Harvard University, Cambridge, MA 02138, USA

Abstract

Bacillus subtilis is a remarkably diverse bacterial species – capable of growth within diverse environments including the gastrointestinal tracts of animals. Microarray-based comparative genomic analyses have revealed that members of this species also exhibit considerable genome diversity. The identification of strain-specific genes might explain how *B. subtilis* has become so broadly adapted. This goal – to identify ecologically adaptive genes – may soon be realized with the imminent release of several new *B. subtilis* genome sequences. As we embark upon this exciting new era of *B. subtilis* comparative genomics we review what is currently known about this species' ecology and evolution.

Where do we find *Bacillus subtilis*?

Bacillus subtilis can be isolated from myriad environments – terrestrial and aquatic – making it appear as though this species is ubiquitous and broadly adapted to grow in diverse settings within the biosphere. However, like all members of the genus *Bacillus*, *B. subtilis* is capable of forming highly resistant dormant endospores in response to nutrient deprivation and other environmental stresses [1]. These spores are easily made airborne and dispersed by wind [2, 3]. Thus, spores might migrate long distances, land in a given environment but never germinate there. Considering that the traditional methods for isolating *B. subtilis* require that the organism be in its spore form, there is no guarantee that when a strain is isolated from a particular environment it was actually growing at that location. Thus, to date, the question of where *B. subtilis* grows remains largely unanswered.

Does *B. subtilis* actually grow in soil or is this a place where spores accumulate until they are once again presented with conditions propitious for their germination and proliferation? The use of fluorescent anti-bodies to distinguish vegetative and spore forms of *B. subtilis* in diverse soil samples [4] revealed that the organism was most often in its vegetative form when associated with decaying organic material [5]. Further support for the idea that *B. subtilis* can lead a saprophytic lifestyle comes from experiments in which spores were inoculated into artificial soil microcosms saturated with filter-sterilized soluble organic matter extracted from soil [6]. Under these conditions the spores not only germinated but the vegetative cells proliferated for several days until they once again sporulated, likely in response to nutrient depletion. Soon after germination the cells formed bundled chains that moved on the surface in a flagella-independent fashion [6]. Interestingly, a similar transition to growth as bundled chains is observed during the early stages of biofilm development under laboratory conditions [7] (Box 1).

B. subtilis can also grow in close association with plant root surfaces. In the laboratory, when *B. subtilis* was inoculated on the roots of *Arabidopsis thaliana* growth of biofilms was observed

*Corresponding author: Phone: (617) 432-1776, Fax: (617) 738-7664, rkolter@hms.harvard.edu.

[8, 9]. In addition, *B. subtilis* can be isolated, in greater numbers than most other spore-forming bacteria, from the rhizosphere of a variety of plants [10–13]. There is evidence that through these associations *B. subtilis* may promote plant growth [13]. Possible explanations for this growth promotion are that: (i) *B. subtilis* out-competes other microbes that would otherwise adversely affect the plant, (ii) *B. subtilis* activates the host defense system so that the plant is poised to resist potential pathogens and, (iii) *B. subtilis* makes certain nutrients more readily available to the plant (*e.g.* phosphorous and nitrogen) [14].

Considering that *B. subtilis* is found on and around plants and that many animals consume plants, it is no wonder that this bacterium is often found in feces [15–17]. Passage of *B. subtilis* through animal gastrointestinal (GI) tracts may not be without effects; the idea that *B. subtilis* plays an active role within the GI tract has had anecdotal support for years. In fact, *B. subtilis* has been touted as a probiotic that when ingested has "beneficial" effects, likely by helping to maintain or restore "healthy" bacterial communities in the body [18]. *B. subtilis* is also found in several commercially available fermented food products, including soy beans fermented with *B. subtilis natto* which is popular in Japan and which has been long thought to confer health benefits [19]. But as its role in plant growth promotion, just how *B. subtilis* imparts its probiotic effects is not clear.

Work from recent years has transformed our view of what *B. subtilis* can do within the GI tract of animals. In the past, *B. subtilis* was thought to be an obligate aerobe that simply transited through the mostly anaerobic GI tract as a spore. Therefore, any benefit incurred by its consumption was thought to be due to some intrinsic property of the spore. Recent evidence, however, suggests that *B. subtilis* can complete its entire lifecycle within the GI tract going from spore to vegetative cell and sporulate again [16–18, 20, 21]. In fact, growth within the GI tract must be robust enough such that it can out-compete pathogens like *E. coli* in poultry GI tracts when administered orally [22].

In summary, current data suggest that *B. subtilis's* apparent ubiquity is not solely a consequence of spore persistence in these environments. Instead *B. subtilis* appears to grow in diverse environments including soils, on plant roots, and within the GI tract of animals.

What can genomics teach us about *B. subtilis* ecology?

Today we find ourselves in a golden age of genomics thanks to increasingly facile methods for generating, assembling, and analyzing large amounts of sequence information [23]. We no longer need to rely solely on isolation geography, behaviors in the laboratory, or anecdotal reports to gather a picture of a species' ecology. In addition, we can investigate the genes present or absent in any strain of interest. The identity of the proteins predicted to be encoded in an organism's genome can reveal much about that organism's lifestyle and the habitats where it resides.

The genome sequence of *B. subtilis* 168 has provided many insights into the lifestyles of the organism [24]. Consistent with the view that the bacterium is not a pathogen, no genes coding for known virulence factors were found. Interestingly, the genome encoded numerous pathways for the utilization of plant-derived molecules, bolstering the idea that this species associates intimately with plants [24]. One observation challenged the long-held belief that *B. subtilis* was an obligate aerobe; genes encoding a putative respiratory nitrate reductase were found [24]. This suggested that *B. subtilis* should be able to grow anaerobically using nitrate instead of oxygen as an electron acceptor. Anaerobic growth of *B. subtilis* in the presence of nitrate has since been demonstrated experimentally [25]. The discovery that *B. subtilis* can indeed grow anaerobically further supports the idea that vegetative life within the mostly anaerobic GI tract of animals is feasible.

The genome sequence also revealed that *B. subtilis* has dedicated a relatively large portion of its genome (~4%) to making secondary metabolites. Some of these compounds are potent inhibitors of fungi and bacteria and likely allow *B. subtilis* to compete in the natural environment [14, 15, 26], promote plant growth, and serve as a probiotic.

The limitations of genome sequence from a single laboratory strain

The genome of *B. subtilis* 168 was chosen for sequencing because the laboratory strain had been the workhorse for molecular genetic studies for several decades. That very strength meant that it had been grown under artificial settings for many generations. As a consequence, the *B. subtilis* 168 strain had evolved in ways that improved fitness in the laboratory, a process that is referred to as domestication [7]. But this domestication came at a cost. We now recognize that *B. subtilis* 168 is deficient in a number of traits that are characteristic of wild strains. Among these are surface swarming and the ability to form architecturally complex biofilms (Box 1 Figure 1) [7, 27]. Conversely, *B. subtilis* 168 produces a much higher proportion of cells in the state of genetic competence than do wild strains.

At the same time that investigators began to recognize strain domestication as a common laboratory phenomenon the genomic era delivered a surprise. In some cases the genomes of different strains of a single species were highly conserved while in others the genetic variability was enormous. In fact, in the case of *Escherichia coli*, even though different strains possess identical 16S rRNA gene sequences, strains can harbor more than one thousand strain-specific genes [28]! There seems to be a trend that the amount of differences in gene content observed within a given species correlates with certain features of that species' ecology. Bacterial species with little genome variability appear to occupy few habitats while those with more genomic diversity within strains appear to colonize diverse environments.

Where does *B. subtilis* lie in the spectrum of genomic diversity? Does the genome of *B. subtilis* 168 tell the full tale of this species' biology and ecology? Is there genomic variation among members of this species? And, if so, could this variation explain differences in strain ecology?

Foreshadowing *B. subtilis* genomic diversity

For many years most of the available evidence concerning genotypic variation among different *B. subtilis* isolates came from the assessment of phenotypic variation, principally strain-to-strain variation in the ability to make various antibiotics [26, 29]. It wasn't until the 1990s that loci other than 16S rRNA genes were examined among multiple strains [30–32]. These studies revealed that *B. subtilis* was not nearly as genetically monomorphic as its pathogenic relative, *B. anthracis* [33]. One such survey used restriction fragment length polymorphisms (RFLP) of three housekeeping genes as markers for genetic diversity among strains isolated from geographically distant locations [30]. The results revealed that these strains were clearly phylogenetically separate from other recognized species of the genus *Bacillus*, but they themselves fell into two distinct phylogenetic groups [30]. This robust phylogenetic separation called into question the assignment of *B. subtilis* as a single species. In other words, did the strains from both phylogenetic clusters belong to the species *B. subtilis* or was there enough variation to reclassify one of these groups as a distinct species within the genus *Bacillus*? Using "classical" methods for bacterial species assignments [34], including DNA re-association analysis, it was concluded that the two groups exhibited sufficient "relatedness" to be kept within the same species, but were different enough to warrant subspecies classification [35]. Thus, strains of *B. subtilis* were divided into subspecies *B. subtilis* subsp. *subtilis*, containing the sequenced strain *B. subtilis* 168, and *B. subtilis* subsp. *spizizenii* [35].

Analyses involving DNA-reassociation kinetics also gave indications that there was more genetic diversity among members of this species than what was found by nucleotide variation at conserved sites [35]. The results suggested that a large percentage of the DNA of each strain's genome was strain-specific. However, the identities of these strain-specific regions were entirely unknown. Could the identities of genes within these variable regions focus and/or expand our view of *B. subtilis's* ecology?

Microarray-based comparative genomic hybridization analyses

Ideally, to begin to answer the foregoing question one would seek to identify and compare all of the genes harbored by each strain. But even though whole genome sequencing has become an increasingly feasible option for such an analysis, it is still not a quick or inexpensive undertaking. However, the available *B. subtilis* 168 genome sequence did provide an opportunity to explore genome variation among strains at much lower cost. Using an oligonucleotide microarray designed to represent each of *B. subtilis* 168's predicted coding sequences, it was possible to query closely related strains for variation in each of *B. subtilis* 168's genes. This technique, called microarray-based comparative genomic hybridization (M-CGH) is simply a DNA re-association method that provides more detailed information about which genes are contributing to lowered re-association values. DNA from strains that either lack or possess a divergent copy of a *B. subtilis* 168 gene will not hybridize as well as the DNA from *B. subtilis* 168 to that gene-specific oligonucleotide. The relative hybridization of a strain's DNA can be easily assessed by measuring variation in fluorescence intensity at each gene spot when the *B. subtilis* 168 and test strains' genomes are differentially labeled with fluorescent nucleotides.

Such analyses were performed using a collection of diverse strains from both subspecies groups [36]. The results from this study revealed that 30% of *B. subtilis* 168's predicted coding sequences were cumulatively absent or divergent in the strains tested [36]. Not surprisingly, strains that were more closely related to *B. subtilis* 168 (within the *subtilis* subspecies) exhibited less total gene diversity relative to those in the other subspecies, consistent with the RFLP and DNA re-association data.

Where is genome diversity localized? To answer this question, knowledge of the extent of synteny among strains is needed. While there was only one *B. subtilis* genome sequence available, a high degree of synteny among *B. subtilis* strains is to be expected given the observed synteny between the *B. subtilis* 168 and the recently published *B. licheniformis* ATCC 14580 genome sequences [37, 38]. Assuming that synteny among *B. subtilis* strains is high, it seems that genomic diversity among this species is not localized to only a few areas within the genome. Rather, it is distributed along the entire genome. In summary, based on the M-CGH analyses there are very few large stretches of genomic DNA that do not have some possibility of variation.

M-CGH analysis reveals regions of variability among wild strains of *B. subtilis*

Within these distributed regions of diversity were some genes that, given previous phenotypic and biochemical observations, came as no surprise. These included genes that encode for the synthesis of secondary metabolites 26, 39, teichoic acid 40, and the adaptive response to alkylation DNA damage [41]. The M-CGH analysis revealed that there was also variability in nearly all "functional" categories of genes, some of which could prove ecologically relevant by changing (expanding or limiting) the environments in which these strains can live. Divergence was observed in genes that encode for the uptake and breakdown of carbohydrates and amino acids (*e.g.* xylose and glutamine) as well as a number of cell surface-associated proteins - including those involved in environmental sensing [36]. The observed variability

among these loci, and others like it, suggests that certain metabolic and/or environmental-monitoring capabilities may not be required for *B. subtilis'* life in all environments.

It is equally informative to determine which genes exhibit limited or no variability. Presumably these highly conserved loci would encode proteins that are selected for in all environments inhabited by the species. As expected, nearly all of the genes that had been previously shown to be essential under laboratory conditions in *B. subtilis* 168 [42] were invariable among the *B. subtilis* strains examined [36]. Also, a very large fraction of the sporulation genes were conserved. This is not surprising given that all of the strains from the M-CGH study were originally isolated as spores. It is interesting to note, however, that many of *B. subtilis* 168's germination genes exhibited divergence. This suggests that the cues for reinitiating growth may not be the same in all environments.

Genes involved in biofilm formation were highly conserved [36]. Life within matrix-associated multi-cellular communities appears to be a universally important ecological trait for this species. However, there are reports that there is some strain-to-strain variation among conserved loci that can affect the outcome of this developmental process [43, 44]. This strain-to-strain variation was not detected in the M-CGH analyses because it involved minor sequence changes in conserved genes and regulatory regions. The noted allelic variation may thus have been the consequence of laboratory domestication alone and not necessarily reflective of variation among wild isolates.

As referred to above, *B. subtilis* is also noted for its ability to become naturally competent for transformation, i.e. the ability to take up and recombine extra-cellular DNA into its genome [45]. The M-CGH analyses revealed that all of the competence machinery identified in *B. subtilis* 168 was highly conserved except for one operon. The three-gene *comPQX* operon is involved in the synthesis, processing, and recognition of an extra-cellular signal that is required for the initiation of competence [46]. *comPQX* had been previously recognized as variable among strains of *B. subtilis* [47–50]. It was further demonstrated that the observed genetic variation also resulted in functional variation such that different strains produced and recognized different variants of the extra-cellular signal to the exclusion of others [47–50]. Considering that competence signal recognition is a population-density-dependent phenomenon, *B. subtilis* strains likely become competent only when their own numbers are high. This would suggest that genetic transfer via transformation would occur most often with DNA from "self".

What are the drivers of diversity/evolution in this species?

How does genome diversity come about? Mutagens as well as DNA replication and repair errors can introduce mutations into a genome. If a mutation is neutral or confers an advantage for life in a given environment, that mutation may become fixed within a population and eventually come to predominate. Though this mechanism for genetic change unquestionably occurs in nature, it is not the primary driver of evolution among bacterial species [51]. Instead, horizontal gene transfer (HGT), via transduction, conjugation or transformation, is thought to play the most important role in this process [51]. Consistent with this notion, the *B. subtilis* 168 genome sequence revealed that a large portion of this strain's genome might have arisen by HGT [52, 53]. And, perhaps not surprisingly, many of the divergent genes (~40%) among the strains examined by M-CGH were located in these regions [36].

Among the genes predicted to have been horizontally transferred many are clustered and bear the hallmarks of phage integration, suggesting that they were gained by transduction. A recent study reported that phage integration could account for as much as 16% of the predicted HGT regions in *B. subtilis* 168's genome [53]. This suggests that, like many other bacterial species examined to date [51, 54], phages are playing a role in the evolution of this species. Whether

phages are actually shaping the ecology of *B. subtilis* via the introduction of novel loci that could be used to explore or reside within different environments is yet to be conclusively determined. Although the presence, within these phage elements, of genes that encode for antibiotic synthesis and detoxification strongly suggests that they could serve such a purpose.

Plasmids mediate gene transfer via conjugation and thus play a role in bacterial evolution [51]. A survey of plasmid diversity among natural isolates of *B. subtilis* estimated that only ~10% of strains harbor these extra-chromosomal elements [32]. All of the plasmids identified appeared to be highly homologous, probably all sharing the same basic replicon [32]. This is different from the account of plasmid diversity in other bacterial species, such as *E. coli* [55, 56]. There is no evidence to suggest that *B. subtilis* plasmids confer any benefit, perhaps explaining their low occurrence among natural *B. subtilis* populations as well as their genetic homogeneity [32]. Although there is report of conjugation between *B. subtilis* strains in soil microcosms [57], it seems unlikely that conjugation is an important driver in the evolution of this species.

Finally, it appears that transformation may indeed help drive the evolution of *B. subtilis*. Under laboratory conditions, strains can take-up and recombine exogenously added genomic DNA from relatives [58]. This can occur even between subspecies although the number of recombinants goes down as relatedness decreases, a phenomenon termed sexual isolation [58]. Also early experiments using sterilized soil microcosms monitored what happened when differentially "marked" variants of strains were mixed [59]. Such exchange was observed even between different species, e.g. *B. subtilis* and *B. licheniformis* [60]. However, the results observed were likely biased by the choice of strains as both laboratory strains used are known to be much more highly transformable then wild strains. The "hybrid species" recombinants were also unstable suggesting that the results may not be relevant to what is occurring in nature. It does appear, however, that wild populations of *B. subtilis* do indeed recombine their genes in nature [30]. How this exchange is mediated – by transformation, transduction or conjugation – is yet to be determined.

In summary, *B. subtilis* is a widely adapted bacterial species, capable of growing within myriad environments including soil, plant roots and the GI tracts of animals. The *B. subtilis* 168 genome sequence has been an important tool in aiding our understanding of how growth within some of these environments is possible. It is now clear, however, that the *B. subtilis* 168 genome does not tell the entire story. M-CGH analyses have revealed great variability among the genes of different members of the species.

Though intriguing, the results of M-CGH give us an incomplete picture. For instance, M-CGH cannot forecast what, if any, genes are present within regions of divergence that are not already found within the *B. subtilis* 168 genome. However, we are poised to answer this question. Whole genome sequences from select representatives of both *B. subtilis* subspecies will soon be available, revealing the identities of genes within these regions of divergence. It will be interesting to see whether some of these genes prove to be ecologically significant and whether they broaden our view of this organism's habitat and the adaptations it has acquired to propagate in diverse environments. Ultimately, the new genome sequences, in conjunction with the M-CGH data, will likely prove powerful in increasing our understanding of *B. subtilis'* ecology and evolution.

Box 1

Biofilm formation by *Bacillus subtilis*

It is now broadly recognized that bacteria have the widespread capacity to form surface-associated multicellular aggregates, commonly referred to as biofilms [61–63]. Within biofilms the constituent cells are embedded in matrices composed of diverse extra-cellular

polymeric substances [61]. Although the benefits afforded microbes during life in these structures are not well understood, biofilm-associated microbes exhibit marked metabolic and physiological differences compared to their planktonic brethren, including decreased susceptibility to antimicrobial agents [64].

Bacillus subtilis has been a model organism for the study of biofilm formation [7, 43, 65–67]. Through a combination of genetic and biochemical approaches, both the structural and regulatory components necessary for the development of these multi-cellular communities have been identified in strain NCIB3610 [7, 66–69] (Figure 1, left), a possible progenitor of the sequenced *B. subtilis* 168 [36] (Figure 1, right). In short, the extra-cellular matrix that holds the biofilm together is comprised of both a protein and polysaccharide component encoded by the *yqxM* and *eps* operons, respectively [66]. Although not essential in *B. subtilis* NCIB3610, γ-polyglumate has also been shown to participate as a component of the extra-cellular matrix in biofilms of some strains of *B. subtilis* [43, 44]. The expression of the *yqxM* and *eps* operons is controlled by a complex regulatory circuitry involving a core regulatory duo, SinI and SinR – the activities of which are subject to upstream regulators including Spo0A. The ability to make complex biofilms like that of *B. subtilis* NCIB3610 appears conserved among wild strains of this species. All of the genes identified in NCIB3610 also appear conserved among strains examined by M-CGH [36].

Acknowledgments

Work in our laboratories on *B. subtilis* biofilms and genome diversity is funded by grants from the National Institutes of Health GM18568 to R.L. and GM58213 to R.K. A.M.E. was the recipient of a postdoctoral fellowship from the National Institutes of Health (GM072393).

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Figure 1.
B. subtilis colony morphologies. *B. subtilis* NCIB3610 (left) and *B. subtilis* 168 (right) spotted onto MSgg agar, a biofilm-inducing medium commonly used in the laboratory to study biofilm development [7]. Note the difference in contoured texture of each strain's surface; *B. subtilis* NCIB3610 exhibits much more complex colony architecture than does the sequenced and domesticated strain, *B. subtilis* 168. Scale bar = 1 cm.