The temperature-adaptive fatty acid content in *Bacillus simplex* strains from 'Evolution Canyon', Israel

Johannes Sikorski,^{1,2} Evelyn Brambilla,¹ Reiner M. Kroppenstedt¹ and Brian J. Tindall¹

Correspondence

Johannes Sikorski

johannes.sikorski@dsMZ.de

¹Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), D-38124 Braunschweig, Germany

²Department of Environmental Microbiology, Helmholtz Center for Infection Research, Inhoffenstrasse 7, D-38124 Braunschweig, Germany

Exploring the evolutionary response of *Bacillus simplex* strains to the slope-specific habitats of 'Evolution Canyon' I and II, Israel, we report here on presumably adaptive differences in fatty acid (FA) content that correlate with one particular feature of the habitats, temperature difference. These two canyons represent similar ecological sites, separated by 40 km, in which the orientation of the sun yields a strong sun-exposed and hot 'African' south-facing slope versus a rather cooler and mesic-lush 'European' north-facing slope within a distance of only 50–400 m. Among 131 strains, which are identical in their 16S sequences, those assigned genetically to the 'African' ecotypes express phenotypically generally more high-temperature-tolerance-providing iso-branched FAs than strains assigned to the 'European' ecotypes when grown at 20 °C, 28 °C and 40 °C. Conversely, 'European' lineages express larger amounts of low-temperature-tolerance-providing anteiso-branched and non-saturated FAs when grown at the same temperatures. Moreover, 'African' ecotypes show a stronger adjustment of their high- and low-temperature-tolerance-providing FAs in response to low temperatures, which suggests that, as a result of temperature adaptation, 'African' and 'European' ecotypes have evolved different reaction norms within their phenotypic plasticity response. Thus, bacterial adaptive microevolution may include such multigenic and highly complex organs as the bacterial cell membrane. The results contribute to our understanding of the speciation process among the 'Evolution Canyon' *B. simplex* ecotypes.

Received 18 December 2007

Revised 3 March 2008

Accepted 15 April 2008

INTRODUCTION

While there have been regular debates as to the nature of prokaryote species (Spratt *et al.*, 2006), little attention has been given to the details of the speciation process itself (Cohan & Perry, 2007). A major driving force of speciation is adaptation by natural selection, which operates at the level of the phenotype (Mayr, 1959, 1997, 2004). Two types of phenotypic changes, which are not mutually exclusive, need to be distinguished here. First, phenotypic plasticity is the ability of a genotype to produce more than one phenotype when exposed to different environments (Garland & Kelly, 2006; Pigliucci, 2005), which is a

transient change. This, however, should not be confused with the second type of change, namely adaptation, which is the movement of a population towards a phenotype that best fits the current environment (Orr, 2005). Thus, adaptation is a process that occurs over many generations, i.e. on a geological timescale.

Most modern bacterial population biology studies are entirely genotype based, generally done by analysis of DNA sequences, and no attempt is usually made to explain the consequences of the sequence differences for the organism in its natural environment. Although the genome codes for all the potential of the organism, it is becoming increasingly clear that we are still a long way from being able to accurately predict the biology of the organism from its DNA sequence (Patridge & Ferry, 2006; White, 2006). A study on microevolution in prokaryotes should therefore not just limit itself to mostly DNA-sequence-based studies, in order to identify habitat-associated genetic groups, but should also attempt to understand the emergence of such

Abbreviations: ai-, anteiso-; FA, fatty acid; i-, iso-; NFS, north-facing slope; SFS, south-facing slope.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are EU305743–EU306135.

Six supplementary figures and two supplementary tables are available with the online version of this paper.

genetic groups due to potential ecological influences, i.e. to identify phenotypes that would correlate with the environment (Sikorski & Nevo, 2007; Sikorski, 2008).

To make a comprehensive study of microbial microevolution, adaptation and speciation in environmental systems, a model population of *Bacillus simplex* (approx. 950 strains) from environmental sites termed 'Evolution Canyons' I and II (EC), Israel, was recently established (Sikorski & Nevo, 2005). ECI (Lower Nahal Oren, Mount Carmel) and ECII (Lower Nahal Keziv, western Upper Galilee) are separated by 40 km and represent similar examples of the same ecological canyon system, with sharply contrasting interslopes in each, separated by only 50–100 m at the bottom and 400 m at the top (Fig. 1a, b) (Nevo, 1995, 1997, 2001). The open park forest of warm-xeric, tropical, 'African' savanna-like, south-facing slopes (SFSs) receive up to eightfold more solar radiation than the north-facing slopes (NFSs) (Pavlicek *et al.*, 2003). Consequently, they are warmer, drier, and spatiotemporally more heterogeneous and fluctuating, and are environmentally distinct from the green, lush, temperate, cool-mesic, 'European' NFSs (Pavlicek *et al.*, 2003). By using genotypic data (randomly amplified polymorphic

DNA fingerprinting and DNA sequences) Sikorski & Nevo (2005) showed, among a set of approximately 950 strains of *B. simplex*, the emergence of evolutionary lineages (genetic clusters) that predominantly reside on either the SFS (lineages A, Ba, C, and D) or the NFS (lineages B, Bae and E), suggesting a specific correlation of the distribution of strains with either the 'African' or 'European' habitat type (Fig. 1c). Moreover, it was shown that the proposed potentially strong migrational exchange between the two canyons (separated by 40 km) and the two slopes (100–400 m) is strongly overridden by the contrasting selective environmental pressure exerted by the two slope types, thereby suggesting ongoing speciation processes (Sikorski & Nevo, 2005). While these genetic studies indicated selection to have taken place, there was no convincing evidence of which specific phenotype(s) could have participated in the slope-specific adaptation. Recently, in a detailed study on 131 representative strains which are identical in their 16S sequences, it was suggested that the differential heat stress on the two slopes could be one such important evolutionary driver (Sikorski & Nevo, 2007). Strains assigned to 'African' lineages were shown to have a higher growth rate than 'European' lineages in Luria–Bertani broth at 43.25 °C (the upper temperature limit of

Fig. 1. The 'Evolution Canyon' model. (a) Schematic diagram. (b) Cross-section view of 'Evolution Canyon' I, Lower Nahal Oren, Mount Carmel, Israel. (c) Phylogenetic relationship of evolutionary lineages of the *B. simplex* metapopulation ($n=995$ strains) as estimated by RAPD (random amplified polymorphic DNA)-PCR and DNA sequences of three genes from up to 27 representative strains (Sikorski & Nevo, 2005). The numbers in parentheses indicate the number of representative strains from the respective evolutionary lineages A–E. Light grey indicates the SFS, and dark grey the NFS. The vertical size of the light or dark grey boxes reflects the relative numbers of strains within each evolutionary lineage, as estimated by the initial RAPD-PCR study (Sikorski & Nevo, 2005). GL1 and GL2 indicate the two major genomic lineages (Sikorski & Nevo, 2005). (d) Indication of the position of 131 representative isolates studied phenotypically previously (Sikorski & Nevo, 2005, 2007) and also in this study (FA analysis and DNA sequence analysis of three genes). (e) Neighbour-joining tree using the concatenated sequences of partial sequences of the *rpoB*, *uvrA* and *gapA* genes. A detailed analysis of the single-gene phylogenies is given in Supplementary Fig. S2. The bar indicates the percentage nucleotide dissimilarity. (f) Genealogy of the clonal relationship of the strains based on the same gene sequence set as in (e). The tree depicts the consensus tree from eight independent runs of ClonalFrame. The arrows and asterisks in (e) and (f) indicate conflicting results within the single-gene phylogenies (see text and Fig. S2). The bar indicates the time in coalescent units.

growth) (Sikorski & Nevo, 2007). Thus, 'African' lineages are presumably better adapted to higher temperatures, a strong climatic feature of the SFS. The less high-temperature-tolerant 'European' strains would probably be at a selective disadvantage when transferred to the hotter SFS slope.

Cell membranes are essential in defining cellular life (Konings, 2006); they are highly complex but also highly dynamic structures, precisely adjusted by the types of fatty acids (FAs), which are essential components of cell membrane lipids (phospholipids, aminolipids, glycolipids, triglycerides, etc.) (Epand, 2005; Gawisch, 2005; Lewis & McElhaney, 2005; Zhang & Rock, 2008). In the Gram-positive aerobic spore-formers, what was traditionally known as the genus *Bacillus*, iso- (i-) and anteiso- (ai-) branched FAs are the dominant components (de Mendoza *et al.*, 2002; Kaneda, 1977). i-FAs are characterized by a methyl group at the second-but-last C-atom of the carbon chain, whereas in ai-FAs the methyl group is located at the third-but-last C-atom. The type of branching (i- or ai-) is dependent on the type of primer molecule at the initiation of the FA synthesis (see Supplementary Fig. S1, available with the online version of this paper). Additionally, unbranched and unsaturated FAs (in lower concentrations) may be present. The type of FA composition is one of the major factors contributing to membrane structure and function. In simple terms, bacteria generally respond to increasing or decreasing temperature by altering their FA composition in a predictable fashion. In the case of organisms with predominantly i- and ai-FAs an increase in growth temperature will generally result in an increase in the ratio of i-FAs to ai-FAs. If the growth temperature decreases, then the ratio of ai- to i-FAs will increase (Kaneda, 1977; Russell, 1989; Zhang & Rock, 2008). These responses are clearly of importance to the survival of the organism (the cell) on a short-term temporal scale. While temperature-dependent changes in the FA and polar lipid composition of individual strains is well documented (Kaneda, 1977, 1991), thereby addressing phenotypic plasticity (Garland & Kelly, 2006; Pigliucci, 2005), there are few studies on groups of strains that may be members of the same species but from different habitats (ecospecies), which would address real evolutionary adaptation via a constitutive, non-transient change in FA expression. Here, we report significant differences in the FA content of 131 strains of *B. simplex*. These are the same strains that have been previously studied with respect to mutation rate and UV-C survival (Sikorski & Nevo, 2005), and temperature-dependent growth rates (Sikorski & Nevo, 2007). We show that there is a correlation of FA content with genetically determined subgroups (evolutionary lineages, ecotypes) and with the original habitat, the 'African' or the 'European' slope, thereby arguing that the different temperatures on the slopes of 'Evolution Canyon' presumably have a long-term (i.e. on a geological scale) influence on the FA composition of the different evolutionary lineages. This could have facilitated adaptation to the

different temperature regimes of SFS and NFS and thereby accordingly reinforced speciation.

METHODS

PCR amplification and sequencing. A part of the *rpoB* gene was amplified and sequenced using primers rpoB-F (5'-AGGTCAAC-TAGTTCAGTATGGACG-3') and rpoB-5R (5'-ATCAAGAGTCC-GACGATC-3'). The sequencing was performed using primers rpoB-F, rpoB-5R, rpoB-2F (5'-CCGCTGATGACTGAAACAGG-3'), rpoB-3F (5'-GCTGATGACTGAAACAGGTAC-3'), rpoB-6F (5'-GCTGAGCCGAAGTATTCTGTGG-3'), rpoB-7F (5'-CCTTGGATTY-GGAACWGATC-3'), rpoB-2R (5'-CCTGTTTCAGTCATCAGCGG-3'), rpoB-3R (5'-GTACCTGTTTCAGTCATCAGC-3'), rpoB-4R (5'-GTAATTTCTCGGTGCGGTCG-3'), rpoB-5R (5'-ATGAAGAGT-CCGACGATC-3'), rpoB-7R (5'-GATCWGTTCCRAATCCAAGG-3') and rpoB-8R (5'-CGGWGAGCGYACTAATTGGG-3'), yielding altogether 871 bases of sequence. A part of the *uvrA* gene was amplified using primers uvrA-1F (5'-ATGTCCGCATTCTGTG-TAAC-3') and uvrA-1R (5'-TGAAGTCTCTCTTGAAGC-3') and sequenced using primers uvrA-2F (5'-CAGTGAACATCATGCTT-GTCC-3') and uvrA-2R (5'-TATTGRGAACGTCGGCTC-3'), yielding altogether 614 bases of sequence. A part of the *gapA* gene was amplified using primers gapA-F (5'-GGGGMTTACC-TTGTTGTTGATGG-3') and gapA-R (5'-TGATACTAGCGGAA-GTTCGC-3'). The PCR products were sequenced using primers gapA-2F (5'-CAATTCGGAATCAAACGCGG-3'), gapA-2R (5'-TGC-TGCCCCAGTTGTGTAG-3') and gapA-3' (5'-TCATACCGCG-TTTGATTCCG-3'), yielding altogether 635 bases of sequence. All sequencing was performed using a Beckman Coulter CEQ Genetic Analysis System capillary sequencer. These sequence data have been submitted to the GenBank database under the accession numbers EU305743–EU306135.

Gene sequence analysis. The partial DNA sequences were subjected to a single-gene sequence (Supplementary Fig. S2) and concatenated gene sequence phylogenetic neighbour-joining analysis with proportional distance using MEGA 3.1 (Kumar *et al.*, 2004) (Fig. 1). For a second assessment of the genealogical relationship of the 131 strains, we used ClonalFrame (Didelot & Falush, 2006), which infers bacterial clonal relationships on the basis of DNA sequences by taking into account both point mutation and homologous recombination. ClonalFrame uses a Bayesian framework assuming a neutral coalescent model. Using all three gene sequences, eight independent runs of ClonalFrame using the default conditions were performed. These were 50 000 generations of the Monte Carlo Markov chain (MCMC), after a burnin of 50 000 generations. Sampling was every 100 generations. The initial values were $\theta=1$, $\delta=0.0001$, $\nu=0.01$ and $R=1$, with allowance for a constant update of these values during the runs. The length of the MCMC runs was sufficient, as indicated by the successful convergence of the results from the independent runs as assessed by the Gelman and Rubin method implemented in ClonalFrame. The results from the eight independent runs were exported as a 50 % majority-rule consensus tree in Newick file format and visualized in MEGA 3.1 using the radial tree option.

FA analysis. All strains were grown on Trypticase Soy Broth Agar (Difco) for 24 h (28 °C and 40 °C) or 40 h (20 °C). The prolonged growth at 20 °C was chosen in order to yield similar colony sizes to those at 28 °C. Harvesting of the cells, saponification, methylation and extraction were done precisely as recommended for taxonomic evaluation by the Sherlock Microbial Identification System (MIDI; <http://www.midi-inc.com>). The samples were analysed on an Agilent Technologies 6890N gas chromatograph. The raw data are given as the percentage of a specific FA among all FAs. The data were analysed

for taxonomic information by the TSBA40 method of the Sherlock MIS software. Further data analysis was done with Statistica 6.0, using standard non-parametric tests as indicated in Table 1 or the text. Initial experiments showed the FA patterns across each three independent replicates of six representative strains to be highly reproducible (Supplementary Fig. S5). Therefore, for the total of 131 strains and at all temperatures, the FA extractions were done only once.

RESULTS

Genealogical relationship of 131 representative strains based on partial sequences of three genes

The initial genetic characterization of the population was based on genetic fingerprinting patterns (all strains) and gene sequences from a limited number of strains ($n=21-27$) (Sikorski & Nevo, 2005). In order to verify and improve the genetically determined population structure, the partial sequences of three genes (*rpoB*, *uvrA* and *gapA*) from all 131 strains were phylogenetically analysed. Overall, the genealogical structure of the 131 strains based on the partial sequence of three genes (Fig. 1e, f) fits well with the previously determined structure (Fig. 1c). However, a major difference was observed. The initial genetic fingerprinting patterns (Sikorski & Nevo, 2005) suggested that lineage Ba was nested within lineage B, and Bae was nested in Ba (Fig. 1c). In contrast, the three gene sequences from 131 strains could not distinguish Bae from B strains, and Ba appeared to be an independent evolutionary lineage not nested within B (Fig. 1e, f). Therefore, in this work, the former Bae strains (Sikorski & Nevo, 2005) were included in lineage B. Also, based on a series of phylogenetic reconstructions on the single-gene and concatenated gene sequences (Supplementary Fig. S2), three strains (marked with an asterisk in Fig. 1e, f) could not unambiguously be affiliated to any of the lineages and were therefore excluded from further lineage-specific comparisons of the FA contents. In total we observed a robust clustering of the strains into the assigned evolutionary lineages and a robust separation of genomic lineages GL1 and GL2 (Fig. 1,

Fig. S2). In GL1, however, we could not determine a robust phylogenetic relationship of lineages A, B, Ba and C to each other. Therefore, in downstream statistical evaluations (Table 2), we treated the GL1 lineages as equivalent groups.

The observed average proportional nucleotide polymorphisms are 0.018, 0.014 and 0.02 for *gapA*, *rpoB* and *uvrA*, with maximum values of 0.046, 0.029 and 0.049, respectively. In *gapA* and *rpoB*, this translates each into only a single amino acid change in a single strain. For *uvrA*, the average and maximum proportional amino acid distances are 0.0038 and 0.02, with K_a/K_s values of all strains below 1.

FA content at 20 °C, 28 °C and 40 °C

At 28 °C, most FAs were either unbranched, or i- or ai-branched. The dominant FA at 28 °C, as averaged over 131 strains, was ai-15:0 (approx. 56 %), followed by i-15:0 (approx. 15 %) (Table 1). All other FAs were in the range of 0.5 % to approx. 5.8 %. Three different mono-unsaturated FAs were present, in a total of approx. 6 % (Table 1). A change in incubation temperature led to substantial changes in the fatty acid content (Fig. 2, Supplementary Table S1). At the lower temperature of 20 °C the unsaturated FAs increased by 2.5–3.7-fold, yielding approximately 17 % of the total FAs (Fig. 2a). The relative concentration of all unbranched or i-branched FAs decreased significantly. Additionally, ai-15:0 and ai-17:0 decreased, although not as strongly as the i-branched or most unbranched FAs (Fig. 2a). At the higher temperature of 40 °C the i-branched FAs increased in their relative amount, whereas the ai-branched and unsaturated FAs decreased (Fig. 2b). At the same time the proportion of unsaturated FAs was significantly reduced. These results are consistent with what is known about the change of branched-chain and saturated/unsaturated FA content in response to growth temperature changes (Kaneda, 1977; Russell, 1989). Therefore, iso-branched FAs were grouped together as ‘high-temperature-tolerance-providing’ FAs, whereas the anteiso-branched and unsaturated FAs were

Table 1. Summary statistics of the fatty acid content (%) of all 131 strains at 28 °C

Fatty acid	Mean	Median	Minimum	Maximum	Standard deviation	Range
ai-15:0	56.31	56.44	36.43	62.96	3.29	26.53
i-15:0	15.60	15.50	10.13	24.38	2.51	14.25
i-14:0	5.85	5.52	3.21	15.34	1.62	12.13
i-16:0	4.22	4.06	2.46	7.70	0.94	5.24
16:0	3.85	3.84	1.94	5.48	0.69	3.54
16:1 ω 11c	3.00	2.90	1.89	5.97	0.57	4.08
ai-17:0	2.66	2.64	1.06	4.35	0.59	3.29
16:1 ω 7c alcohol	2.45	2.31	1.51	5.96	0.65	4.45
14:0	1.68	1.66	1.04	2.55	0.32	1.51
i-17:0	1.60	1.55	0.68	2.49	0.37	1.81
15:0	1.04	1.01	0.52	2.66	0.28	2.14
i-17:1 ω 10c	0.56	0.55	0.00	1.00	0.15	1.00

Table 2. Values of statistical significances of the differences of lineages as depicted in Fig. 3(a–j)

Figure	Feature	SFS vs NFS (<i>P</i> -value)*	Differences within GL1 (lineages A, B, Ba, C)	Within GL2: lineage D vs E (<i>P</i> -value)*
3(a)	High-temperature-tolerance-providing FAs, ratio 20 °C vs 28 °C	0.0019	$P < 0.0001$ † A differs significantly from B at the 0.05 level of significance‡ A differs significantly from Ba at the 0.01 level of significance‡ B differs from Ba at the 0.05 level of significance‡	0.76
3(b)	High-temperature-tolerance-providing FAs, 20 °C	0.059	$P = 0.0239$ † A differs significantly from Ba at the 0.05 level of significance‡ B differs from Ba at the 0.05 level of significance‡	0.26
3(c)	High-temperature-tolerance-providing FAs, 28 °C	0.0043	$P = 0.109$ †	0.031
3(d)	High-temperature-tolerance-providing FAs, 40 °C	0.067	$P = 0.06$ †	0.0075
3(e)	High-temperature-tolerance-providing FAs, ratio 40 °C vs 28 °C	0.67	$P = 0.027$ † Ba differs from C at the 0.05 level of significance‡	0.88
3(f)	Low-temperature-tolerance-providing FAs, ratio 20 °C vs 28 °C	0.16	$P = 0.0001$ † A differs significantly from B and Ba at the 0.05 level of significance‡	0.20
3(g)	Low-temperature-tolerance-providing FAs, 20 °C	0.22	$P = 0.0243$ † A differs significantly from Ba at the 0.05 level of significance‡	0.39
3(h)	Low-temperature-tolerance-providing FAs, 28 °C	0.015	$P = 0.324$ †	0.019
3(i)	Low-temperature-tolerance-providing FAs, 40 °C	0.040	$P = 0.0334$ † A differs from C at the 0.05 level of significance‡	0.0057
3(j)	Low-temperature-tolerance-providing FAs, ratio 40 °C vs 28 °C	0.33	$P = 0.02$ † A, Ba and B each differ from C at the 0.05 level of significance‡	0.029

**P*-value obtained from the Mann–Whitney U test.

†*P*-value obtained from the Kruskal–Wallis H test.

‡Statistical significance of differences between individual lineages was obtained from the post-hoc Tukey–Kramer test, using as input the rank-sum values obtained from the Kruskal–Wallis H test.

grouped together as ‘low-temperature-tolerance-providing’ FAs. The i- and ai-FAs, at 28 °C, sum up to a total of ~86 %.

The distribution of high- and low-temperature-tolerance-providing FAs differs among ‘African’ and ‘European’ lineages at 20 °C 28 °C and 40 °C

The ‘African’ strains as a group produce significantly more high-temperature-tolerance-providing FAs at 28 °C than the ‘European’ strains (Fig. 3c, Table 2). This qualitative pattern is also true for the individual ‘African’ lineages in GL1 (although not statistically significant, Table 2) and in GL2 (statistically significant, Table 2). Moreover, the same qualitative pattern is observed also at 20 °C (Fig. 3b, with the exception of lineage A) and 40 °C (Fig. 3d, with the exception of lineage C).

Conversely, the ‘European’ strains as a group produce significantly more low-temperature-tolerance-providing FAs at 28 °C than the ‘African’ strains (Fig. 3h, Table 2). This is also true for GL2 lineages (D differs significantly from E, Table 2), but only partially for GL1 strains. The larger amounts of low-temperature-tolerance-providing FAs in ‘European’ strains is also observed at 20 °C and 40 °C, with the exception of lineage A at 20 °C (Fig. 3g) and lineage C at 40 °C (Fig. 3i). Details on individual FA types are given in Supplementary Fig. S3.

In summary, over a wide range of temperatures (20 °C, 28 °C and 40 °C), ‘African’ lineages produce generally more high-temperature-tolerance-providing FAs, and ‘European’ lineages produce more low-temperature-tolerance-providing FAs. The consistency of these results over a temperature range of 20 °C (20–40 °C) strongly suggests a stable manifestation of constitutive expression levels of

Fig. 2. Relative increase or decrease of FAs at 20 °C and 40 °C as compared to 28 °C. The values are the mean ratio values of 131 strains as depicted in Fig. 1(c). The high-temperature-tolerance-providing i-branched FAs are in light grey, the low-temperature-tolerance-providing ai-branched and unsaturated FA are in black. The straight-chain FAs are in dark grey.

temperature-relevant FA types, even to some extent buffering the substantive changes due to the phenotypic plasticity response (at 20 °C and 40 °C, Fig. 2). This difference in FA pattern is most distinct at the presumably non-stressful temperature of 28 °C (there is no difference in growth of 'African' versus 'European' strains at 30 °C: Sikorski & Nevo, 2007).

It must be noted that Fig. 3 provides the sum of the high- and low-temperature-tolerance-providing FAs. The individual FA types, however, may differ in their individual expression levels across different lineages. For example, at 28 °C, the 'African' lineage D expresses a significantly

higher amount of the high-temperature-tolerance-providing FA group of i-15:0 and i-17:0 ($P=0.00042$, Mann-Whitney U, MWU, Fig. S3m) than the 'European' lineage E. The same qualitative pattern is observed for the other high-temperature-tolerance-providing FA group of i-14:0 and i-16:0, although the difference is less pronounced and non-significant ($P=0.36$, MWU, Fig. S3h). At 40 °C, the situation is the opposite. Lineage D expresses significantly larger amounts of i-14:0 and i-16:0 ($P=0.00021$, MWU, Fig. S3i) than lineage E; however, in the group of i-15:0 and i-17:0 FAs, surprisingly the 'European' lineage E expresses larger amounts of these FAs (although non-significantly, $P=0.089$, MWU, Fig. S3n)

Fig. 3. Box-whisker plots of absolute values and temperature-dependent ratios of FAs. (b, c, d, g, h, i) Absolute summed values (%) of high-temperature-tolerance-providing iso-branched and low-temperature-tolerance-providing ai-branched and unsaturated FAs at 20 °C, 28 °C and 40 °C. (a, e, f, j) Temperature-dependent ratios of the absolute FA values. The black and white checked boxes refer to all 131 strains, white boxes are 'African' strains (lineages A, Ba, C, D), and grey boxes are 'European' strains (lineages B and E). Lineages A, B, Ba, C, and lineages D, E represent the main genomic lineages GL1 and GL2, respectively (see Fig. 1). Open circles and filled squares indicate mean and median values, respectively. The broad boxes indicate the 25 % to 75 % quartiles, the thinner boxes indicate the standard deviation, and the whiskers depict the maximum and minimum values.

than lineage D. Thus, lineages D and E differ fundamentally in how strongly they upregulate from 28 °C to 40 °C the amount of these two different but both high-temperature-tolerance-providing FA groups. Lineage D more strongly upregulates the i-14:0 and i-16:0 FAs ($P=0.014$, MWU, Fig. S3j), whereas lineage E significantly more strongly upregulates the i-15:0 and i-17:0 group ($P=0.000001$, MWU, Fig. S3o). However, despite such differences in individual FA groups, the 'African' lineage D expresses at both 28 °C and 40 °C significantly larger amounts of the high-temperature-tolerance-providing FAs than does the 'European' lineage E (Fig. 3c, d, Table 2).

The temperature-dependent ratio of high- and low-temperature-tolerance-providing FAs differs among 'African' and 'European' lineages

It is necessary to determine the temperature-dependent ratios of high- and low-temperature-tolerance-providing FAs, as these data can give insights into (a) how well strains may adjust their cell membrane contents during acute temperature shifts and (b) how this trait may change on microevolutionary, geological timescales.

As a group, 'African' strains have a significantly lower 20 °C to 28 °C ratio of high-temperature-tolerance-providing FAs (Fig. 3a, Table 2). Thus, at 20 °C, the 'African' lineages deplete better than 'European' lineages the rather unnecessary high-temperature-tolerance-providing FAs. This pattern holds also for the individual GL1 lineages. In GL2, lineage D values are only marginally and non-significantly smaller than in lineage E (Fig. 3a, Table 2). Additionally, as a group, 'African' strains have a higher (although not significantly) 20 °C to 28 °C ratio of low-temperature-tolerance-providing FAs (within GL1, A has a significantly larger ratio than B and Ba, Table 2) (Fig. 3f). Thus, at 20 °C, the 'African' lineages build up relatively greater amounts of the necessary low-temperature-tolerance-providing FAs than do the 'European' lineages.

There is no difference between 'African' and 'European' lineages in the 40 °C to 28 °C ratio of high- or low-temperature-tolerance-providing FAs (Fig. 3e, j). Within GL1, there are some non-significant differences between individual lineages, but there are no tendencies for either 'African' or 'European' lineages to show higher ratios (Fig. 3e, j). However, in GL2 and for low-temperature-tolerance-providing FAs, lineage D has a significantly lower ratio than lineage E (Fig. 3j). Details of individual FA types are given in Supplementary Fig. S3.

Chain length ratios in dependence on FA type and incubation temperature

The length of FA chains derived from the same primer molecule (Supplementary Fig. S1) depends on the enzymes participating in the elongation cycle (de Mendoza *et al.*, 2002). Thus, determining the chain length ratios across the 131 strains (which are in fact 131 natural mutants) with

respect to (a) type of FA and (b) incubation temperature may give valuable insights into the plasticity of the elongation cycle in evolutionary terms.

First, across different types of FAs, the ratios of short to long chains differs strongly, ranging from approximately 0.5 (14:0 versus 16:0) to 23 (ai-15:0 versus ai-17:0) (averaged over 131 strains, Supplementary Table S2). Second, within a FA type, the ratios may differ quite strongly between different evolutionary lineages (Supplementary Fig. S4). Third, with either decreasing (20 °C) or increasing (40 °C) temperature, as compared to 28 °C, the ratio of short to long chains across all FA types studied here increases (Table S2). This is significant at 20 °C for 14:0 versus 16:0 and for i-14:0 versus i-16:0 (Table S2) and at 40 °C, for all branched types of FA (Table S2). Fourth, interestingly, at a constant temperature, strains keep their relative position in the population with respect to the chain length ratios at different FA types very constant. For example, strain I3b2o (labelled '1' in Fig. 4) has among all 131 strains at 28 °C the smallest ai-15:0 versus ai-17:0 ratio (value of 14.0) (Fig. 4a). The same strain also has the smallest i-15:0 versus i-17:0 ratio in the population at 28 °C; however, at a different absolute value (6.32; Fig. 4a). By contrast, strain II1b16 (labelled '6' in Fig. 4) has at 28 °C the highest chain length ratio in the population for both ai-15:0 versus ai-17:0 (value of 51.42) and i-15:0 versus i-17:0 (value of 19.09). In total, across all 131 strains, this correlation is highly positive and significant (Fig. 4a) and holds also for other FA type comparisons, as long as the comparison is across the same temperature (Fig. S4). Fifth, this strong and significant positive correlation at 28 °C (Fig. 4a) is, among the same FA types, also observed at 20 °C (Fig. 4b) and at 40 °C (Fig. 4c). However, most interestingly, the rank order of strains is not the same at the different temperatures. As an example, this is visualized with six strains labelled '1' to '6' in Fig. 4(a–c), which significantly change their relative positions in the population at different temperatures; the six strains were chosen semi-randomly in order to reflect the diversity range of Fig. 4(a). In general, this strong relaxation of the positive correlation across different temperatures is true for all 131 strains, as shown by the examples in Fig. 4(d–i). This indicates that temperature has a significant influence on the enzymes of the elongation cycle, in the way that chain length ratios across different strains change to different extents with respect to temperature shifts. Fig. 4 shows these relationships for the i-15:0, i-17:0, ai15:0 and ai-17:0 FAs. Fuller data, including other FA types, are shown in Supplementary Fig. S4.

Lack of correlation of growth at high temperature (43.25 °C) with the amount of high-temperature-tolerance-providing FAs at 28 °C for all 131 isolates

Within the same set of strains, we compared previously published temperature-relevant physiological results

Fig. 4. Correlation of chain length ratios in dependence on type of FA and temperature. Values on the *x*- and *y*-axes denote the ratio of short to long FA chains of the indicated FA type at the indicated incubation temperature. The Spearman rho (*R*) value and respective *P* value in the upper left corner of the individual panels (a) to (i) refer to the full set of 131 strains (small black dots). The respective values in the upper right corners refer to the subset of six strains marked with numbers 1 to 6 in circles. These are strains I3b20 (SFS), I6a10s (NFS), I3c8o (SFS), I1b3o (SFS), I3c8o (SFS) and I13b10 (SFS), respectively. A coloured version of this figure, allowing for SFS versus NFS differentiation among all depicted strains, is available as supplementary material (Fig. S6).

(Sikorski & Nevo, 2007) to the FA results presented here. All 'African' lineages show higher growth rates at 43.25 °C (the upper temperature limit of growth) (Sikorski & Nevo, 2007) and generally higher amounts of high-temperature-tolerance-providing FAs (Fig. 3), suggesting a weakly positive although non-significant correlation of both as adaptively identified traits (Fig. 5a). On a strain-to-strain level, however, there is no correlation between both traits (Spearman $R=0.07$, $P=0.43$; all strains) (Fig. 5b).

DISCUSSION

Phenotypic adaptation, including reaction norms, of FA content to the habitat temperature difference of the slopes

Based on mainly genetic data, evidence for a habitat-dependent distribution of the very closely related group of *B. simplex* strains was found previously, which suggested

Fig. 5. Correlation plot of growth at high temperature (43.25 °C) and amount of heat-adaptive FAs at 28 °C of all 131 isolates. The growth values represent the integral of the growth curve between 60 and 540 min in rich liquid medium. Basically, the higher the growth value, the better is the growth; see Sikorski & Nevo (2007) for details. (a) Means and standard deviations of the strains of the evolutionary lineages as given in Fig. 1(e). (b) The letters indicate the evolutionary lineage as depicted in Fig. 1(e). 'African' strains are given in black (lineages A, Ba, C, D); 'European' strains are given in grey (lineages B and E). Lineages A, B, Ba, C, and lineages D, E represent the main genomic lineages GL1 and GL2, respectively (see Fig. 1).

adaptation to the microclimatically contrasting slopes of 'Evolution Canyon' (Sikorski & Nevo, 2005). It would appear that temperature is a major force accounting for this observation (Sikorski & Nevo, 2007).

On the basis of differences in FA content, we provide here a second phenotypic line of evidence for temperature-adaptive evolution to the different temperature regimes on the NFS and SFS slopes of 'Evolution Canyon'. As in both dominant groups of GL1 and GL2 the 'African' lineages express higher amounts of i-FA than the respective

'European' counterpart lineages, this could be an example of convergent evolution. Additionally, 'African' lineages respond more strongly than 'European' lineages in adjusting their amounts of high- and low-temperature-tolerance-providing FAs to cold temperatures (Fig. 3a, f). Thus, beyond a rather simple although important constitutive microevolutionary adjustment of FA expression levels to the environments of the slopes, it appears that 'African' lineages additionally have evolved within the immediate temperature-stress response (phenotypic plasticity) a different reaction norm from that of 'European' lineages, probably as a result of the long-term temperature adaptation to the 'African' and 'European' slopes. Interestingly, this pattern coincides with the more pronounced level of positive correlation of growth at high (43.25 °C) and low (20 °C) temperatures among 'African' lineages (see Figs 5 and 7 of Sikorski & Nevo, 2007). Both patterns could perhaps be related to the larger daily and annual temperature range of the 'African' slope, expressing the clearly hotter and also the colder (albeit less strong) temperature extremes (Pavlicek *et al.*, 2003). Both patterns suggest 'African' lineages to evolve more strongly than 'European' lineages in the direction of generalists.

The molecular nature of this adaptation is still unclear. Also, it is not known if the phenotypically observed convergent evolution (adaptation) in the phylogenetically clearly separated lineages of 'African' A, Ba, and C versus D and 'European' B versus E is due to the same underlying molecular mechanism. It was previously found that the two *Bacillus subtilis* FabH homologues, which carry out the initial condensation reaction of FA biosynthesis, show different affinities for the i- and ai-branched-chain acyl-CoA primers as substrates. FabH1 did have a slight preference for the anteiso- precursor, 2-methylbutyryl-CoA, while FabH2 was more active with the iso-precursors, isobutyryl-CoA and isovaleryl-CoA (Choi *et al.*, 2000). Each FabH homologue showed its own characteristic specificity level for either precursor molecule (Choi *et al.*, 2000). Thus, the *B. simplex* FabH homologues could be suitable candidates to account for some of the divergent expression of i- and ai-branched FA in 'African' and 'European' lineages.

The temperature-dependent plasticity of the elongation cycle as a potential target for selection

The ratio of the chain lengths is not fixed (Supplementary Table S2), despite the fact that most probably the same set of enzymes participates in the elongation of all FAs (de Mendoza *et al.*, 2002). This suggests that the affinity of the enzymes for their substrates depends not only on the length of the molecule itself, but also on the type of primer molecule (Supplementary Fig. S1). The analysis of the population-wide diversity reveals that any mutation affecting the chain length ratio does this apparently equally well across all types of FA, as indicated by the very strong positive correlation across all combinations of FA types, as

long as the temperature is constant (Fig. 4a–c, Supplementary Fig. S4). However, temperature might affect the substrate affinity of different mutations in a different way (Fig. 4 a–c, compare the relative positions of strains labelled ‘1’ to ‘6’; Fig. 4d–i). This leads to some extensive plasticity of chain length ratios across different strains and temperatures, not, however, affecting the chain length ratios with respect to the type of (primer) molecule itself. Adaptation by natural selection could act on this diversity, as we note that the temperature-dependent chain length ratios of several FA types change significantly in the direction of a lower short-to-long chain ratio at 20 °C and 40 °C, as compared to 28 °C (Supplementary Table S2). The biological significance of this, in terms of both phenotypic plasticity and real evolutionary adaptation, is currently unclear, especially as there is no significant tendency of either SFS- or NFS-associated lineages to express different FA chain length ratios at different temperatures (Supplementary Table S2).

Insights into characteristics of the natural selection process within *B. simplex* from ‘Evolution Canyon’

It appears that natural selection has substantively shaped the population structure of *B. simplex* in ‘Evolution Canyon’ (Sikorski & Nevo, 2005, 2007).

Although the qualitative interpretation of the FA phenotype shows a strong positive and adaptive correlation of the FA phenotype with the habitat, and although in several cases this is supported by significant quantitative effects (Table 2), the fuzziness of the data cannot be overlooked. The descriptive standard deviation error bars show strong overlap between ‘African’ and ‘European’ lineages (Fig. 3). From the point of view of data presentation, we would have wished to present more clear-cut data. However, the observed fuzziness represents the biological reality and by this suggests that the evolutionary separation of phenotypes lags behind the clear-cut separation observed at the level of single genes (Fig. 1e, f; Supplementary Fig. S2). This was also already observed in the phenotype of growth at 43.25 °C (the upper temperature limit of growth) (Sikorski & Nevo, 2007). On the population biology level, this may reflect the fact that the observed ‘African’ and ‘European’ lineages are in the early stages of evolutionary separation. It also suggests that strains, e.g. those from the ‘African’ slope, which do not express one (or both) of the adaptive phenotypes described up to now are not immediately selected against. Thus, selection appears to remove only the least-adapted strains, and does not necessarily favour only the best-adapted strains. On the molecular level, this may be explained by the fact that the phenotypes studied here and previously (Sikorski & Nevo, 2007) are multi-genetic traits. This gives a large range of mutational targets (within a single strain) for creating the potentially adaptive diversity (in a population) on which selection may then act.

Other characteristics of the natural selection process of adaptation to the SFS and NFS are less clear. For example, does adaptation occur predominantly by selection for survival or by selection for reproductive success? In *B. simplex*, the selection-for-survival hypothesis has not yet been solidly tested. The previously performed experiments for UV-C survival (Sikorski & Nevo, 2005) do not reflect natural *in situ* conditions. A more natural survival stress could be phagocytic predation, protection against which may be mediated by an intact spore coat (Klobutcher *et al.*, 2006). Two lines of evidence suggest that the latter (selection for reproductive success) at least participates. First, ‘African’ lineages express the more positive and significant correlation of growth rates at both stressful high (43.25 °C) and low (20 °C) temperatures (Sikorski & Nevo, 2007). Second, ‘African’ lineages differ from ‘European’ lineages in their reaction norm by responding more strongly to low temperatures while adjusting their composition of high- and low-temperature-tolerance-providing FAs (Fig. 3a, f). Both traits suggest ‘African’ lineages to evolve more strongly than ‘European’ lineages in the direction of a generalist, in the sense of being able to cope relatively better with short-term changes (i.e. within the phenotypic plasticity response) to both high and low temperatures. We would relate such a direction of evolution to the selection for reproductive success rather than to selection for survival.

Concluding remarks

Summarizing, the genetic data clearly document the existence of groupings within populations from different habitats. However, genetic data as such, even if available at the extent of full genome sequences, would not necessarily indicate the type and direction of phenotypic changes on which selection operates (Mayr, 1959, 1997, 2004). This study, together with earlier ones (Sikorski & Nevo, 2005, 2007), therefore strengthens the necessity for population-biology-based phenotypic analyses, together with genetic and ecological studies, when approaching the microevolution of bacteria (Sikorski, 2008). By demonstrating here a second phenotype correlating with the habitat-dependent distribution of genotypic groups, FA content, this study additionally supports the notion of bacterial species as being represented by ecotypes (Cohan & Perry, 2007; Koepfel *et al.*, 2008; Sikorski, 2008).

ACKNOWLEDGEMENTS

We thank our DSMZ colleagues for fruitful discussions. J.S. thanks the Deutsche Forschungsgemeinschaft (DFG) for financial support (grant SI 1352/1-1).

REFERENCES

Choi, K.-H., Heath, R. J. & Rock, C. O. (2000). β -Ketoacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. *J. Bacteriol.* **182**, 365–370.

- Cohan, F. M. & Perry, E. B. (2007). A systematics for discovering the fundamental units of bacterial diversity. *Curr Biol* **17**, R373–R386.
- de Mendoza, D., Schujman, G. E. & Aguilar, P. S. (2002). Biosynthesis and function of membrane lipids. In *Bacillus subtilis and its Closest Relatives: from Genes to Cells*, pp. 43–55. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Didelot, X. & Falush, D. (2006). Inference of bacterial microevolution using multilocus sequence data. *Genetics* **175**, 1251–1266.
- Epand, R. M. (2005). Role of membrane lipids in modulating the activity of membrane bound enzymes. In *The Structure of Biological Membranes*, pp. 499–509. Edited by P. L. Yeagle. Boca Raton: CRC Press.
- Garland, T., Jr & Kelly, S. A. (2006). Phenotypic plasticity and experimental evolution. *J Exp Biol* **209**, 2344–2361.
- Gawisch, K. (2005). The dynamics of membrane lipids. In *The Structure of Biological Membranes*. Edited by P. L. Yeagle. Boca Raton: CRC Press.
- Kaneda, T. (1977). Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol Rev* **41**, 391–418.
- Kaneda, T. (1991). Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol Rev* **55**, 288–302.
- Klobutcher, L. A., Ragkousi, K. & Setlow, P. (2006). The *Bacillus subtilis* spore coat provides “eat resistance” during phagocytic predation by the protozoan *Tetrahymena thermophila*. *Proc Natl Acad Sci U S A* **103**, 165–170.
- Koeppel, A., Perry, E. B., Sikorski, J., Krizanc, D., Warner, A., Ward, D. M., Rooney, A. P., Brambilla, E., Connor, N. & other authors (2008). Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. *Proc Natl Acad Sci U S A* **105**, 2504–2509.
- Konings, W. N. (2006). Microbial transport: adaptations to natural environments. *Antonie Van Leeuwenhoek* **90**, 325–342.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lewis, R. N. A. H. & McElhaney, R. N. (2005). The mesomorphic phase behaviour of lipid bilayers. In *The Structure of Biological Membranes*, pp. 53–120. Edited by P. L. Yeagle. Boca Raton: CRC Press.
- Mayr, E. (1959). Where are we? *Cold Spring Harb Symp Quant Biol* **24**, 409–440.
- Mayr, E. (1997). The objects of selection. *Proc Natl Acad Sci U S A* **94**, 2091–2094.
- Mayr, E. (2004). *What Makes Biology Unique? Considerations on the Autonomy of a Scientific Discipline*. New York: Cambridge University Press.
- Nevo, E. (1995). Asian, African and European biota meet at “Evolution Canyon” Israel: local tests of global biodiversity and genetic diversity patterns. *Proc R Soc Lond [Biol]* **262**, 149–155.
- Nevo, E. (1997). Evolution in action across phylogeny caused by microclimatic stresses at “Evolution Canyon”. *Theor Popul Biol* **52**, 231–243.
- Nevo, E. (2001). Evolution of genome-phenome diversity under environmental stress. *Proc Natl Acad Sci U S A* **98**, 6233–6240.
- Orr, H. A. (2005). The genetic theory of adaptation: a brief history. *Nat Rev Genet* **6**, 119–127.
- Patridge, E. V. & Ferry, J. G. (2006). WrbA from *Escherichia coli* and *Archaeoglobus fulgidus* is an NAD(P)H:quinone oxidoreductase. *J Bacteriol* **188**, 3498–3506.
- Pavlicek, T., Sharon, D., Kravchenko, V., Saaroni, H. & Nevo, E. (2003). Microclimatic interslope differences underlying biodiversity contrasts in “Evolution Canyon”, Mt. Carmel, Israel. *Isr J Earth Sci* **52**, 1–9.
- Pigliucci, M. (2005). Evolution of phenotypic plasticity: where are we going now? *Trends Ecol Evol* **20**, 481–486.
- Russell, N. J. (1989). Functions of lipids: structural roles and membrane function. In *Microbial Lipids*, pp. 279–365. Edited by C. Ratledge & S. G. Wilkinson. London: Academic Press.
- Sikorski, J. (2008). Populations under microevolutionary scrutiny: what will we gain? *Arch Microbiol* **189**, 1–5.
- Sikorski, J. & Nevo, E. (2005). Adaptation and incipient sympatric speciation of *Bacillus simplex* under microclimatic contrast at “Evolution Canyons” I and II, Israel. *Proc Natl Acad Sci U S A* **102**, 15924–15929.
- Sikorski, J. & Nevo, E. (2007). Patterns of thermal adaptation of *Bacillus simplex* to the microclimatically contrasting slopes of “Evolution Canyon” I and II, Israel. *Environ Microbiol* **9**, 716–726.
- Spratt, B. G., Staley, J. T. & Fisher, M. C. (2006). Introduction: species and speciation in micro-organisms. *Philos Trans R Soc Lond B Biol Sci* **361**, 1897–1898.
- White, R. H. (2006). The difficult road from sequence to function. *J Bacteriol* **188**, 3431–3432.
- Zhang, Y.-M. & Rock, C. O. (2008). Membrane lipid homeostasis in bacteria. *Nat Rev Microbiol* **6**, 222–233.

Edited by: M. Hecker