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Odile Bouvet, Emmanuelle Bourdelier, Jeremy Glodt, Olivier Clermont, Erick Denamur. Diversity of the auxotrophic requirements in natural isolates of *Escherichia coli*. *Microbiology*, 2017, 163 (6), pp.891-899. 10.1099/mic.0.000482. hal-04560828

HAL Id: hal-04560828

<https://hal.science/hal-04560828>

Submitted on 26 Apr 2024

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Diversity of the auxotrophic requirements in natural isolates of *Escherichia coli*

Odile Bouvet,¹ Emmanuelle Bourdelier,¹ Jeremy Glodt,¹ Olivier Clermont¹ and Erick Denamur^{1,2,*}

Abstract

Isolates of *Escherichia coli*, except *Shigella*, are generally prototrophic; they do not require any growth factors to grow in mineral medium. However, a nicotinic acid requirement is common among B2 phylogroup STc95 O18 *E. coli* clone strains. Nicotinic acid is a precursor of nicotinamide adenine dinucleotide (NAD), an essential molecule that plays central role in cellular metabolism. The defect in NAD synthesis of these strains is due to alterations in *de novo* biosynthesis pathway *nadB* gene. Here, by studying growth on minimal medium with glycolytic (glucose) or gluconeogenic (pyruvate or succinate) substrates as the carbon supply in a large panel of *E. coli* natural isolates representative of the species diversity, we identify an absolute nicotinic acid requirement in non-STc95 strains due in one case to a *nadA* inactivation. The growth on glucose medium of some extraintestinal pathogenic *E. coli* strains belonging to various non-O18 B2 phylogroup STc95 clones is restored either by aspartate or nicotinate, demonstrating that the nicotinic acid requirement can also be due to an intracellular aspartate depletion. The auxotrophic requirements depend on the carbon source available in the environment. Moreover, some strains prototrophic in glucose medium become auxotrophic in succinate medium, and conversely, some strains auxotrophic in glucose medium become prototrophic in succinate medium. Finally, a partial depletion of intracellular aspartate can be observed in some prototrophic strains belonging to various phylogroups. The observed more or less significant depletion according to isolates may be due to differences in tricarboxylic acid cycle enzyme activities. These metabolic defects could be involved in the adaptation of *E. coli* to its various niches.

INTRODUCTION

Thanks to its metabolic versatility [1], *Escherichia coli* is able to adapt to a variety of environments. *E. coli* is a commensal of the gastrointestinal tract in warm-blooded animals, but it can also possess virulence factors involved in intestinal (intestinal pathogenic *E. coli*, InPEC) or extraintestinal (extraintestinal pathogenic *E. coli*, ExPEC) infection. In its secondary habitat, soil, water and sediments, *E. coli* can survive and sometimes grow [2]. This diversity of lifestyles is achieved through a high degree of genome plasticity, with gene losses and gains by horizontal gene transfer as well as other genomic modifications, like DNA rearrangements and point mutations [3, 4]. The population structure is predominantly clonal [5], with the clear delineation of seven main phylogroups (A, B1, B2, C, D, E and F) [6]. The *Shigella*, despite their name, are in fact highly specialized clones of *E. coli* [7, 8]. They are found only in humans where they behave as obligatory intracellular pathogens. Analysis of *Shigella* genomes revealed the presence of a large virulence

plasmid but also many insertion sequences and pseudogenes, leading to the loss of several catabolic functions [9].

It has been shown that besides non-synonymous mutations, gain of function by horizontal gene transfer and gene amplification, adaptation to a new environment can involve the accumulation of beneficial null mutations. Loss-of-function of enzymatic and regulatory proteins can result in reorganization of metabolic and regulatory networks [10]. These mutations can also render the strain auxotrophic, i.e. unable to synthesize an essential compound for growth. The genome-scale metabolic reconstruction works of multiple *E. coli/Shigella* strains have permitted the prediction of strain-specific auxotrophies [11]. This study and others suggest that biosynthetic functions for vitamins (niacin or vitamin B₃, thiamin or vitamin B₁, folate or vitamin B₉) and amino acids (methionine, tryptophan and leucine) [12–15] are more frequently lacking.

Niacin or nicotinic acid (Na) is a precursor of nicotinamide adenine dinucleotide (NAD). NAD is an essential molecule

Received 1 February 2017; Accepted 2 May 2017

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Keywords: *Escherichia coli*; auxotrophy; nicotinate; aspartate.

Abbreviations: Na, nicotinic acid; PCA, principal component analysis; ROS, reactive oxygen species.

One supplementary figure and six supplementary tables are available with the online Supplementary Material.

implicated in numerous redox reactions in cellular metabolism. *E. coli* synthesizes NAD either *de novo* or from salvage pathways (Fig. 1). The *de novo* biosynthesis pathway starts with the oxidation and condensation of aspartate to quinolinic acid catalysed by L-aspartate oxidase (NadB) and quinolate synthase (NadA). The quinolate phosphoribosyltransferase (NadC) then converts quinolinic acid to nicotinic acid mononucleotide. In the absence of functional enzymes, exogenous Na can be used to produce NAD. The last two enzymes of the *de novo* pathway, NadD and NadE, are also common to the pyridine ring salvage pathway. They convert the nicotinic acid mononucleotide to NAD. Cells may also recycle by-products of NAD consumption. In *E. coli*, the major intracellular recycling pathway is the nicotinamide (Nm)/Na pyridine ring salvage pathway. The latter takes precedence over the *de novo* biosynthesis pathway whenever precursors are available in the environment. The level of intracellular NAD is tightly controlled by the tri-functional NadR protein containing the N-terminal helix-turn-helix DNA-binding domain (NadR_R) [16]. Mutations in each of genes *nadA*, *nadB* and *nadC* lead to an auxotrophy for Na [17–19]. All *Shigella* strains require Na for growth in minimal medium and carry non-functional *nadA* and/or *nadB* genes [20]. This dependence is rarely found in enteroinvasive *E. coli* strains that cause a syndrome identical to shigellosis [15]. It is instead more prevalent among cystitis isolates of serotype O18:K1:H7 [14, 21].

In this study, we investigated the effect of three resource environments on the auxotrophic requirements for growth of a large panel of non-*Shigella E. coli* natural isolates, with the aim of having a broader view of nutritional requirements within the species *E. coli*. Our approach was motivated by (i) the huge genomic and metabolic *E. coli* species diversity contrasting with the few available data on auxotrophic requirements, especially for non-*Shigella* strains; (ii) the fact that *E. coli* strains can colonize various niches with different carbon sources; and (iii) the possibility that genomic reconstruction approaches [11] could have missed some complex auxotrophic requirements based on metabolic regulation. The growth capacity in minimal medium with glycolytic (glucose) and gluconeogenic (pyruvate or succinate) carbon sources (Fig. 1) were examined in a range of *E. coli* strains representative of various pathovars and phylogenetic groups of the species. Then nutritional supplementation experiments were realised to determine their auxotrophic requirements in glucose and succinate media. The majority of strains unable to grow in glucose medium are ExPEC of phylogenetic group B2 auxotrophic for nicotinate. However, the growth of some of these strains is restored either by nicotinate or aspartate. This result demonstrates that besides the existence of mutations in the *de novo* biosynthesis pathway genes, the nicotinate requirement can also be due to an intracellular aspartate depletion. Moreover, a partial depletion of intracellular aspartate can

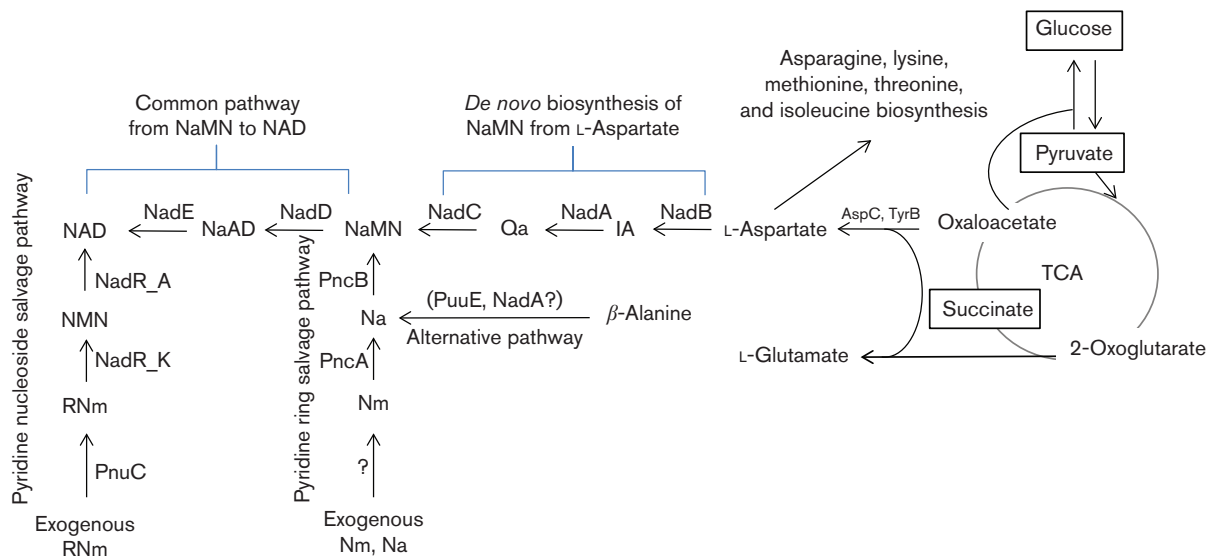


Fig. 1. The *de novo* biosynthesis and salvage pathways of nicotinamide adenine dinucleotide (NAD) in *E. coli*. NAD is synthesized via two major pathways: the *de novo* pathway from aspartate and the salvage pathways. Abbreviations for metabolic intermediates are: IA, imminoaspartate; Qa, quinolinic acid; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; Na, nicotinic acid; Nm, nicotinamide; RNm, ribosylnicotinamide; NMN, nicotinamide mononucleotide. Abbreviations for enzymes are: AspC, aspartate aminotransferase; TyrB, tyrosine aminotransferase; NadB, L-aspartate oxidase; NadA, quinolinate synthetase; NadC, quinolinate phosphoribosyltransferase; NadD, nicotinate-mononucleotide adenylyltransferase; NadE, NAD synthetase; PncA, nicotinamidase; PncB, nicotinate phosphoribosyltransferase; PnuC, ribosyl nicotinamide riboside transporter; NadR_K, ribosylnicotinamide kinase; NadR_A, NMN adenylyltransferase; PuuE, 4-aminobutyrate aminotransferase. TCA stands for tricarboxylic acid cycle. The carbon sources supplied in the growth experiments are boxed: glucose (glycolytic) and pyruvate or succinate (gluconeogenic) substrates.

be observed in some prototrophic isolates. We also show that the auxotrophic requirements can depend on the carbon source available in the environment.

METHODS

Bacterial strains

We studied 43 *E. coli* strains, encompassing 14 commensal strains (seven human strains and seven non-human mammalian strains), 28 pathogenic strains [two strains from animals (one ExPEC and one InPEC), and 26 human strains comprising 19 ExPEC involved in urinary tract infection, newborn meningitis, septicaemia and miscellaneous infections and seven InPEC], and one human asymptomatic bacteriuria (ABU) strain. These strains belonged to various phylogenetic groups (A, B1, B2, D, E and F) and subgroups (STc). The main characteristics of the strains are given Table S1 (available in the online Supplementary Material).

Media and growth

Minimal medium (MM) was composed of the following components: 34 mM NaH_2PO_4 , 64 mM K_2HPO_4 , 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.3 mM MgSO_4 , 40 mM KCl and 1 mM FeCl_3 , pH 7.1. Cells preserved at -80°C were grown overnight at 37°C in the standard lysogeny broth (LB) then in MM supplemented with 10 mM arabinose in order to reduce intracellular metabolite concentrations. Arabinose was chosen as carbon source as it is utilized by the majority of *E. coli* strains and because it does not induce the regulation effects observed with glucose. Cells from this second pre-culture were centrifuged and washed five times with MM and finally re-suspended in MM supplemented with 20 mM glucose, succinate or pyruvate. Suspended cells were washed extensively prior to inoculation to remove trace compounds present in the pre-culture medium. Growth experiments were assayed using 96-well plates and OD_{600} was measured with a Tecan Infinite M200 plate reader. Each well contained 200 μl cell suspension diluted to obtain 2×10^5 bacteria ml^{-1} . Each growth curve was characterized by two parameters: maximal growth rate (μ_{Max}) and the lag time. μ_{Max} was calculated from the logarithm curve of the number of bacteria (N) as a function of time (T), according to the equation $\log_{10} N_2 - \log_{10} N_1 = \mu_{\text{Max}} (T_2 - T_1) / 2.303$. The lag time corresponds to the time taken to reach the μ_{Max} . All experiments were performed at least in triplicate.

Determination of auxotrophic requirements

Nutritional supplementation experiments were realised according to Holliday [22] and Lederberg [23]. Nutritional requirements were screened by the addition to MM of pools containing amino acids, vitamins, purines or pyrimidines. Seventeen pools of growth factors were prepared. Pool 1 ($\mu\text{g ml}^{-1}$): adenine, 10; cytosine, 10; guanine, 10; thymine, 10; uracil, 10. Pool 2 ($\mu\text{g ml}^{-1}$): biotin, 0.002; hypoxanthine, 10; thiamin, 0.002; pyridoxin, 0.1; NAD, 0.1. Pool 3 ($\mu\text{g ml}^{-1}$): L-serine, 20; L-tryptophan, 20; L-alanine, 20; L-cysteine, 20; L-threonine, 20. Pool 4 ($\mu\text{g ml}^{-1}$): sodium thiosulfate, 10; L-methionine, 20; L-arginine, 20; L-aspartate, 20; L-proline, 20.

Pool 5 ($\mu\text{g ml}^{-1}$): L-glutamate, 20; glycine, 20; L-isoleucine, 20; L-lysine, 20; L-valine, 20. Pool 6 ($\mu\text{g ml}^{-1}$): adenine, 10; biotin, 0.002; L-serine, 20; sodium thiosulfate, 10; L-glutamate, 20. Pool 7 ($\mu\text{g ml}^{-1}$): cytosine, 10; hypoxanthine, 10; L-tryptophan, 20; L-methionine, 20; glycine, 20. Pool 8 ($\mu\text{g ml}^{-1}$): guanine, 10; thiamin, 0.002; L-alanine, 20; L-arginine, 20; L-isoleucine, 20. Pool 9 ($\mu\text{g ml}^{-1}$): thymine, 10; pyridoxin, 0.1; L-cysteine, 20; L-aspartate, 20; L-lysine, 20. Pool 10 ($\mu\text{g ml}^{-1}$): uracil, 10; NAD, 0.1; L-threonine, 20; L-proline, 20; L-valine, 20. Pool 11 ($\mu\text{g ml}^{-1}$): riboflavin, 0.5; pantothenic acid, 0.1; folic acid, 0.1. Pool 12 ($\mu\text{g ml}^{-1}$): L-phenylalanine, 20; L-tyrosine, 20; *p*-aminobenzoic acid, 0.1. Pool 13 ($\mu\text{g ml}^{-1}$): nicotinic acid, 0.1; L-ornithine, 20; inositol, 1. Pool 14 ($\mu\text{g ml}^{-1}$): L-leucine, 20; L-histidine, 20. Pool 15 ($\mu\text{g ml}^{-1}$): riboflavin, 0.5; L-phenylalanine, 20; nicotinic acid, 0.1; L-leucine, 20. Pool 16 ($\mu\text{g ml}^{-1}$): pantothenic acid, 0.1; L-tyrosine, 20; L-ornithine, 20; L-histidine, 20. Pool 17 ($\mu\text{g ml}^{-1}$): folic acid, 0.1; *p*-aminobenzoic acid, 0.1; inositol, 1. When testing for growth stimulation by pools, 100 μl each pool were added to 100 μl cell suspension containing 2×10^5 bacteria ml^{-1} in MM supplemented with 20 mM glucose or succinate.

The use of double-entry tables between the pools (P1 to P5) and (P6 to P10) and between the complementary pools (P11 to P14) and (P15 to P17) makes it possible to quickly identify the incriminated growth factor (see Table S2). We have verified that, for each strain showing an auxotrophy by this approach, in independent cultures: (i) no growth was observed when the strain was cultivated with only the carbon source (glucose or succinate); (ii) no growth was observed when the strain was cultivated with only the growth factor at the given concentration; and (iii) growth was observed when the strain was cultivated with the carbon source and the growth factor.

Statistical methods

Differences between means of at least three to nine experiments were evaluated for statistical significance using Tukey's honest significant difference (HSD) test. $P < 0.05$ were considered significant. Data are presented as mean \pm standard deviation. Principal component analysis (PCA) was performed in Excel using the XLSTAT software. Data clustering was done in Excel using the XLSTAT software by agglomerative hierarchical clustering method.

Comparative genomics of the genes coding for the enzymes of the NAD pathways

The genes coding for proteins involved in the *de novo* biosynthesis (*nadA*, *nadB* and *nadC*) and salvage pathways (*pncA*, *pncB*, *pnuC*, *nadR* and *puuE*) of NAD were studied (Fig. 1). When available, gene sequences were extracted from genomes obtained from MaGe [24]. In specific cases and for non-sequenced genomes, genes were PCR-amplified with specific primers (Table S3) and sequenced by classical Sanger technology. Protein multiple sequence alignments were realised using the CLUSTALW program.

RESULTS

Ability to grow on glycolytic and gluconeogenic nutrients as the sole carbon and energy sources

We first grew 43 *E. coli* strains representative of the genetic and lifestyle diversity of *E. coli* (Table S1) in MM with glycolytic (glucose) or gluconeogenic (pyruvate or succinate) substrates as the carbon supply (Fig. 1), using the protocol described in Methods. Using this protocol with extensive washing and a second pre-culture in MM with arabinose, we thoroughly removed metabolites in the culture medium, allowing accurate detection of auxotrophic requirements (see below). For each carbon source, the maximum growth rate (μ_{Max}) as well as the lag time measured as the time to reach the maximum growth rate were determined (Table S4). The relationships between growth parameters of the 43 *E. coli* isolates grown in the three nutrient media are well illustrated by a PCA (Fig. 2). As expected, the diagram reveals a negative relationship between both parameters (lag and μ_{Max}), regardless the carbon source used. A negative correlation indicates the two variables move in opposite directions. Moreover, the positive correlation between lag or μ_{Max} in glucose and pyruvate media indicated those parameters are largely redundant. However, they are not correlated with parameters measured in succinate medium.

Hierarchical clustering of all parameters revealed distinctive patterns among *E. coli* isolates (Fig. 3a). When all parameters are considered, there was a clear separation between

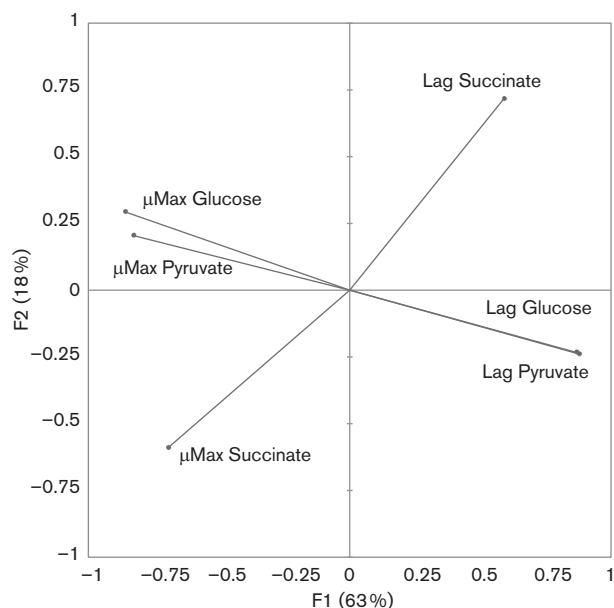


Fig. 2. Principal component analysis (PCA) of growth parameters for 43 *E. coli* isolates in the three culture media. The length and direction of the lines represent the contribution of growth parameters of specific substrates to the first two dimensions. Values in brackets indicate the percentage of explained variance by each dimension.

strains into four clusters, based on trends in their ability to use glucose, pyruvate or succinate as a sole carbon source for growth in MM. The first two related clusters encompassed strains not growing on glucose (except strain DSM 6601) or growing after a very long lag time (strains APECO1 and S88). The first cluster comprised six strains (yellow, see Table S4 for colours) growing on pyruvate and succinate whereas the second one was composed of seven strains (orange) not growing on pyruvate and with variable growth on succinate. The other two related clusters corresponded to strains growing on glucose with a cluster of four strains (blue) growing on pyruvate (except strain 55989) but not on succinate and a cluster comprising the 26 remaining strains (white) growing on pyruvate and succinate (Table S4).

When considering the phylogenetic origin of the strains (Table S1), it can be noted that all the strains except one (E22, B1 phylogroup) not growing on glucose belonged to the B2 phylogroup whereas strains growing on glucose belonged to various phylogroups (A, B1, B2, D and F). The B2 phylogroup strains not growing on glucose were mainly ExPEC (all except ED1a, Asp12e and DSM6601), in agreement with the link between extraintestinal virulence and B2 phylogroup [25].

Auxotrophic requirements of *E. coli* strains

The auxotrophic requirements of cells were then studied, based on the lag time, using a representative panel of 28 strains belonging to the four clusters identified above. The lag time of these strains with and without supplementation were measured in the presence of only two substrates (glucose and succinate), since both glucose and pyruvate substrates give similar growth profiles. Hierarchical clustering of lag time on these two substrates revealed that strains were distributed into four clusters (Fig. 3b), roughly similar to the previous ones based on growth characteristics on the three substrates (Fig. 3a). Thirteen isolates had a short lag time in the glucose and succinate media (Glc+, Suc+), four isolates had a long and short lag time on glucose and succinate media, respectively (Glc–, Suc+), three isolates had a short and long lag time on glucose and succinate media, respectively (Glc+, Suc–) and eight isolates had a long lag time on both media (Glc–, Suc–) (Table S5).

In Fig. 3(c), the hierarchical clustering of lag time in glucose with supplementation well illustrated the nutritional requirements of cells grown in the presence of glucose (Table S5). Nine isolates were unable to grow in glucose medium or grew with a prolonged lag-phase, unless 0.1 μM nicotinate was added to minimal medium. The growth of five of these nicotinate-requiring strains (IAI56, IAI46, IAI60, APECO1 and S88) was also restored by 0.5 mM aspartate or glutamate to a lesser extent (Table S5). On the contrary, aspartate supplementation did not support the growth of three other *E. coli* strains auxotrophic for nicotinate (Asp12e, UTI89 and E22) and only partially restored the growth of strain RS218. Similarly, glutamate supplementation sustained partially growth of *E. coli* Asp12e and UTI89 but was not sufficient for RS218 and E22 (Table S5).

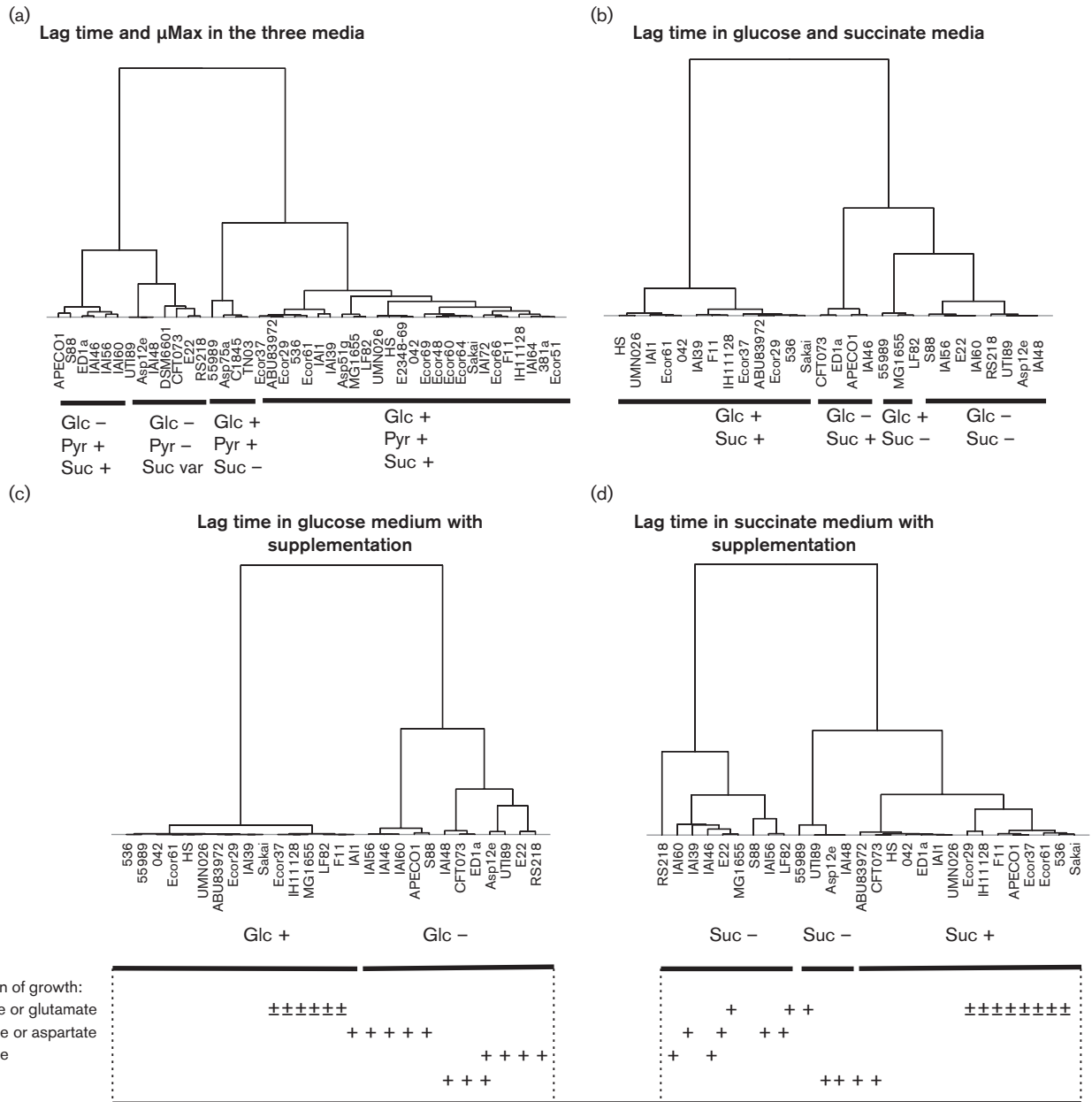


Fig. 3. Hierarchical clustering of growth parameters in different culture media supplemented or not with growth factors. (a) Lag time and μ Max measured for the 43 strains in the three culture media. The + and – signs next to the carbon source indicate growth or no growth, respectively, based on the μ Max values (see exceptions in the text). (b) Lag time measured for 28 strains in glucose and succinate media. The + and – signs next to the carbon source indicate short and long lag time, respectively. Lag time and growth gain measured for 28 strains in glucose (c) and succinate (d) media supplemented or not with growth factors (0.1 μ M nicotinate, 0.5 mM aspartate, glutamate or isoleucine, hypoxanthine). The + and – signs next to the carbon source indicate short and long lag time, respectively. In the bottom part of the figure, the ± sign for the Glc+ and Suc+ strains indicates a decrease of the lag time. For the Glc– and Suc– strains, the + sign indicates a restoration of growth. Glc, glucose; Pyr, pyruvate; Suc, succinate; var, variable.

Finally, the three other strains, *E. coli* IAI48, CFT073 and ED1a differed by their growth factor requirements. *E. coli* IAI48 was auxotrophic for hypoxanthine. Both strains CFT073 and ED1a required aspartate and isoleucine to grow in glucose medium. The sixteen other *E. coli* strains

grew well in the glucose medium without supplementation, however some of them have a relatively long lag-phase. The addition of 0.5 mM aspartate or glutamate reduced the adaptation period of one to two hours for six of these strains (Ecor37, IH1128, MG1655, LF82, F11 and IAI1).

Interestingly, all five strains auxotrophic for nicotinate or aspartate belonged to the B2 subgroup IX/STc95, and within the STc95, to subgroups C, D or D+C [26]. They exhibited O-types 1, 45a and 2a. On the other hand, among the four strains requiring only nicotinate, two strains (UTI89 and RS218) belonged to the B2 subgroup IX/STc95 but to the subgroup B and are of O-type 18, while the two other strains belonged to B2 subgroup IV/STc141, with a O-type 2a (Asp12e) and to the phylogenetic group B1 with a O-type 103 (E22).

We also examined the requirement for growth factors in succinate medium (Table S5). As shown Fig. 3(d), Suc– strains were distributed into two clusters. The first group included nine strains that were auxotrophic for nicotinate (RS218 and IAI39) and/or aspartate (IAI60, IAI46, MG1655 and S88) or glutamate (E22, IAI56 and LF82). The four isolates of the second group (55989, UTI89, Asp12e and IAI48) differed by their growth factor requirements. As in the glucose medium, *E. coli* IAI48 was auxotrophic for hypoxanthine. The growth factor requirements of the three other strains were not identified. Finally, among the Suc+ strains, eight had their growth improved by addition of aspartate or glutamate.

It appears clearly that the behaviour of isolates can vary depending on the carbon source being used. Only four strains of the first group (RS218, IAI 60, IAI 46 and S88) retained the same nutritional requirements in succinate medium as in glucose medium. In the succinate medium, *E. coli* strains IAI 56 and E22 became auxotrophic only for aspartate. In addition, three prototrophic isolates (IAI 39, MG1655 and LF82) in glucose medium became dependent on the presence of aspartate or nicotinate in succinate medium. Moreover, the nutritional requirements of strains Asp12e and UTI89 seemed more complex. The addition of nicotinate was no longer sufficient to restore their growth in the succinate medium. Moreover, the strain 55989 that was prototrophic in glucose medium became auxotrophic. Conversely, three isolates (CFT073, ED1a and APECO1) auxotrophic in glucose medium became prototrophic with succinate. Only IAI 48 remained auxotrophic for hypoxanthine whatever the carbon source.

Multiple alignments of protein sequences of key enzymes in NAD biosynthesis

We investigated further the presence of mutations in eight enzymes involved in the *de novo* and salvage pathways of NAD biosynthesis in a subset of 23 isolates representative of the phylogenetic diversity and of the patterns of observed growth (Table S6). Four of the strains (HS, 042, UMN026 and ABU83972) grow well in the glucose and succinate media whereas 11 strains are auxotrophic for nicotinate in glucose and/or succinate medium (IAI56, IAI46, IAI60, APECO1, Asp12e, UTI89, E22, RS218, IAI39, MG1655 and S88). The genes were present in all the strains, except *puuE* that was absent in the B2 phylogroup strains, and only one truncating mutation was observed in *nadA* in strain E22 (see below). The many enzyme amino acid

changes observed between the strains reflect the evolutionary history of the strains (Table S6). This is confirmed by the fact that phylogenetic trees reconstructed from the nucleotide sequences of the corresponding genes are congruent with the species phylogeny (Fig. S1). Very few amino acid changes were strain-specific, and in this case corresponded mainly to unique strains within a phylogroup (Sakai and IAI39 in the E and F phylogroups, respectively). However, we observed the well-described A28V mutation in the *nadB* gene responsible for the nicotinate requirement of strain UTI89 [21] in strain RS218, which belongs to the same clone (B2-IX/STc95-B-O18) (Table S1), and a nonsense mutation (W299X) was found in *nadA* gene in the E22 B1 phylogroup strain. No clear link between amino acid changes and growth phenotype could be evidenced for the remaining strains.

DISCUSSION

Genome reduction is a typifying feature of bacteria that occur in nutrient-rich or constant environments. This reductive genome evolution often leads to loss of function that render these bacteria auxotrophic. So, their survival is dependent on availability of growth factors in their environment. *Shigella* strains, which are human-specific virulent clones of *E. coli*, represent a perfect illustration of this evolutionary strategy. Several auxotrophic requirements were reported in *Shigella*. Most of them require nicotinate for growth on minimal media [12]. Their defect in NAD synthesis is due to alterations in the *de novo* biosynthesis pathway genes *nadB* and/or *nadA* (Fig. 1) [20], representing a case of convergent evolution [27].

On the other hand, non-*Shigella E. coli* strains are remarkably adapted to a broad diversity of environments, and this ability to occupy these niches is in part due to their unsurpassed metabolic versatility and phenotypic plasticity [1, 28]. *E. coli* is found in the gastrointestinal tracts of humans and vertebrate animals, but it can also adapt to a more nutritionally limited environment, such as the urinary tract. *E. coli* co-metabolize multiple simple sugars via the glycolysis pathway during growth in the intestinal tract. However, *E. coli* EDL933, an intraintestinal pathogen, uses both glycolytic and gluconeogenic nutrients to colonize mice pre-colonized with *E. coli* MG1655 [29]. When extraintestinal pathogenic *E. coli* transit to the urinary tract, they adapt their metabolism to preferentially utilize gluconeogenic carbon sources, like small peptides and amino acids present in urine [30]. Very few data are available on the auxotrophy of natural *E. coli* isolates except the fact that *E. coli* O18:K1:H7 isolates causing urinary tract infections have a nicotinate requirement [14, 21]. These strains belong to the B2 phylogroup, STc95-O18 clone [31] and carried a single A28V mutation in *NadB* [21]. Urine is a complex growth medium that generally provides sufficient nutrients for survival of auxotrophic *E. coli* [32, 33], and *E. coli* UTI89 (STc95-O18) that is defective in synthesis of nicotinate grows well in urine [34].

In agreement with these works, we found that isolates UTI89 and RS218, both belonging to the same STc95-O18 clone, have an absolute requirement for nicotinate for growth in glucose medium due to the A28V mutation (Table S6). Interestingly, this auxotrophy was also found in two non-human strains not belonging to the same clone. Strain E22 is a rabbit intraintestinal pathogen belonging to phylogenetic group B1 in which we detected a non-sense mutation in *nadA*, whereas strain Asp12e is a coypu commensal B2 phylogenetic group-STc141 in which we do not detect any significant mutation in the *de novo* biosynthesis pathway enzymes. Further investigations will be required to identify the mechanism of this nicotinate auxotrophy in isolate Asp12e.

Moreover, we observed different auxotrophic requirements in five other B2 phylogroup ExPEC belonging to non-O18 STc95 clones (IAI56, IAI46, IAI60, APEO1 and S88). Their growth in glucose medium is restored either by aspartate or nicotinate (Fig. 3c). This result rules out the existence of mutation in the *de novo* biosynthesis pathway and demonstrates that the nicotinate requirement can also be due to an intracellular aspartate depletion. Metabolic changes induced by growth on glucose could explain this phenotype [35, 36]. The aspartate is synthesized from oxaloacetate and glutamate, both produced by the TCA cycle (Fig. 1). So, the glucose repression of the TCA cycle that occurs in a cAMP-dependent manner could lead to depletion in aspartate. Aspartate is also the precursor for other amino acids (L-threonine, L-methionine and L-lysine). A deficiency in these amino acids would result in an activation of their biosynthetic pathway and an increase in the aspartate consumption. However, our complementation experiments showed no auxotrophy for these amino acids. The auxotrophic requirements can also depend on the carbon source available in the environment. For example, we observed that nicotinate is not enough to satisfy growth of strains UTI89 and Asp12e in succinate medium. Moreover, some isolates prototrophic in glucose medium become auxotrophic in succinate medium, and conversely, some isolates auxotrophic in glucose medium become prototrophic with succinate certainly by mobilizing other enzymes.

A glucose repression effect on growth is also observed in some prototrophic isolates. Generally, *E. coli* isolates are prototrophic; they do not require any growth factors for growth in minimal medium. However, our study shows that a partial depletion of intracellular aspartate can be observed in some prototrophic strains from various phylogenetic groups (strains Ecor37, IH11128, K-12 MG1655, LF82, F11 and IAI1) grown in glucose medium (Fig. 3c). This more or less significant depletion according to isolates may be due to differences in intracellular concentrations of cAMP. However this aspartate depletion was also detected in succinate medium, thus indicating that other regulators may be involved.

The loss of specific genes could facilitate the adaptation to specific host niches. Studies have shown that auxotrophy-

causing mutations could have a beneficial effect when the focal metabolite is present in the environment. Under these conditions, the mutant saves the biosynthetic cost of the compound. Competitive experiments between auxotrophic mutants and prototrophic wild-type revealed that metabolic auxotrophies promote significantly fitter strains [37–39]. These mutations by disturbing the regulatory and metabolic network of cells lead to changes in metabolic fluxes improving cellular metabolic homeostasis [10, 40, 41]. In *E. coli* auxotrophic for nicotinate, these metabolic rearrangements could help to reduce the rate of intracellular hydrogen peroxide (H_2O_2), especially those carrying a non-functional *nadB* gene. Among the enzymes that form H_2O_2 , the autoxidizable enzyme NadB accounts for about one-quarter of the H_2O_2 generated endogenously in aerobic *E. coli* [42]. Endogenous reactive oxygen species (ROS) such as H_2O_2 are generated continuously in cells grown aerobically. They are responsible for damage to nucleic acids (RNA and DNA), as well as proteins and lipids, leading to cell death [43, 44]. The reorganization of metabolic fluxes generated by glucose could also lead to a reduction in the intracellular ROS level, even in the absence of non-functional gene(s) in the *de novo* biosynthesis pathway. Oxaloacetate is a TCA cycle intermediate required in the biosynthesis of aspartate [45]. The repression of the TCA cycle activity could explain the aspartate (or glutamate) auxotrophy during growth with excess glucose. In *Shigella*, the loss of *nadA* and/or *nadB* genes corresponds to pathoadaptive events. Indeed, the proteins encoded by these genes catalyse the synthesis of quinolinate, an inhibitor of *Shigella* virulence. The expression of *nadA* and/or *nadB* can therefore be deleterious within intracellular niche [20, 46, 47]. Virulence of *Candida glabrata* is also affected by a nicotinate limitation during murine urinary infection. The alteration of intracellular NAD level induces three adhesins, which have roles in adherence to uroepithelial cells and in bladder colonization. *C. glabrata* is a NAD auxotroph found only with the mammalian host [48]. However, no difference was found between the colonization capacity of NAD auxotrophic *E. coli* O18 strains and their prototrophic derivatives in the murine ascending urinary tract infection model [21].

In conclusion, by studying auxotrophy in a large panel of natural isolates of *E. coli*, we showed that although absolute nicotinate requirement due to NAD *de novo* biosynthesis pathway is mostly restricted to the specific B2 phylogroup STc95-O18 clone, nicotinate requirement due to an aspartate intracellular depletion can be observed in a wide range of B2 phylogroup clones. Furthermore, partial aspartate depletion in prototrophic strains is widespread within the species. These metabolic defects could be involved in the adaptation of *E. coli* to its various niches.

Funding information

This work was partly supported by a grant from the 'Fondation pour la Recherche Médicale' to E.D. (Equipe FRM 2016, grant number DEQ20161136698).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Edited by: G. Uden and F. Sargent

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