Variation in Siderophore Biosynthetic Gene Distribution and Production across Environmental and Faecal Populations of Escherichia coli

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Abstract

Iron is essential for Escherichia coli growth and survival in the host and the external environment, but its availability is generally low due to the poor solubility of its ferric form in aqueous environments and the presence of iron-withholding proteins in the host. Most E. coli can increase access to iron by excreting siderophores such as enterobactin, which have a very strong affinity for Fe3+. A smaller proportion of isolates can generate up to 3 additional siderophores linked with pathogenesis; aerobactin, salmochelin, and yersiniabactin. However, non-pathogenic E. coli are also able to synthesise these virulence-associated siderophores. This raises questions about their role in the ecology of E. coli, beyond virulence, and whether specific siderophores might be linked with persistence in the external environment. Under the assumption that selection favours phenotypes that confer a fitness advantage, we compared siderophore production and gene distribution in E. coli isolated either from agricultural plants or the faeces of healthy mammals. This population-level comparison has revealed that under iron limiting growth conditions plant-associated isolates produced lower amounts of siderophores than faecal isolates. Additionally, multiplex PCR showed that environmental isolates were less likely to contain loci associated with aerobactin and yersiniabactin synthesis. Although aerobactin was linked with strong siderophore excretion, a significant difference in production was still observed between plant and faecal isolates when the analysis was restricted to strains only able to synthesise enterobactin. This finding suggests that the regulatory response to iron limitation may be an important trait associated with adaptation to the non-host environment. Our findings are consistent with the hypothesis that the ability to produce multiple siderophores facilitates E. coli gut colonisation and plays an important role in E. coli commensalism.
Introduction

Iron is an essential element implicated in many cellular processes such as DNA replication, energy generation and protection from oxidative stress. When oxygen is present, free ferrous iron (Fe^{2+}) is rapidly oxidised to the insoluble ferric iron (Fe^{3+}). To cope with the reduced bioavailability of ferric iron, bacteria such as *E. coli* have evolved mechanisms to scavenge iron molecules in order to maintain their intracellular iron concentration between 10^{-7} and 10^{-5} M [1]. It has been estimated that in order to survive and multiply in most environments, Gram-negative bacteria require 10^5 to 10^6 Fe^{3+} ions per generation [2]. The main mechanism of ferric iron uptake in *E. coli* involves the synthesis of up to 4 distinct siderophore molecules with very high affinity for iron. After synthesis, siderophores are secreted into the external environment. Siderophore-iron complexes are then imported through specific transporters and degraded to release the iron in the bacterial cytosol [1]. In *E. coli*, siderophores have been described as virulence factors in pathogenic strains and are therefore suggested as targets for antibacterial compounds to limit pathogenic growth [3]. However, their presence in commensal strains highlights the need to reassess the role of siderophores in the general ecology of *E. coli*, which also includes the external non-host environment, where *E. coli* can persist for several weeks [4]. In particular, vegetables, an increasingly recognised secondary reservoir for *E. coli* [5], might represent an iron limited environment where siderophore-mediated iron uptake is an important determinant of bacterial fitness [6–8]. In agreement with this hypothesis, gene expression of the enterobactin and salmochelin siderophores was induced in *Salmonella Typhimurium* in alfalfa root exudates [9].

The diversity in siderophore production displayed by the *E. coli* species suggests that the distribution of siderophores among a population is shaped by the environmental requirements, as with other traits [10,11]. Taking advantage of our recently described GMB collection, which groups 96 environmental isolates from agricultural plants, mainly grown in the UK [11], we compared siderophore production and distribution in these plant-associated strains with *E. coli* isolated from healthy mammalian hosts. We used this approach to investigate the influence of the environment on siderophore production by *E. coli*.

Materials and Methods

Bacterial strains

The plant-associated *E. coli* strains (GMB collection) used in this study have been described previously [11], and mostly comprise strains isolated between 2008 and 2009 in England from the aerial parts of salad crops such as spinach and rocket (76/96). A minority of strains have been isolated from other crops, salad bags and field soil. The ECOR collection includes 61 isolates obtained from the faeces of healthy mammals and 11 isolates from the urine of women with urinary tract infections [12]. The subset of 61 faecal isolates will be referred to as ECOR-F and comprises 29 human and 32 animal isolates mainly isolated in the USA and Europe in the 1980s. The ECOR collection was kindly provided by Beth Whittam (Michigan State University, USA).

Siderophore production

To assess siderophore production *in vitro*, bacterial colonies were grown on chrome azurol S (CAS) [13] plates for 48h at 37°C with glucose as the carbon source. Colonies of siderophore-producing bacteria grown on this medium are surrounded by a yellow or orange halo. The ability to produce siderophores was quantified by measuring the halo diameter using the ImageJ software (http://rsbweb.nih.gov/ij/index.html). To assess any siderophore-specific differences in diffusion that may influence halo size on CAS agar plates, a liquid CAS assay was performed. Single colonies were grown in modified M9 media with shaking for 16h at 37°C [14]. OD_{600}
measurements were performed before cultures were pelleted and supernatants collected [13]. Supernatant from strains positive for siderophore production cause a colour change from blue to orange when mixed with CAS assay solution. This colour change was measured using OD$$_{630}$$ and percent siderophore units calculated and standardised to culture OD$$_{600}$$ [13].

**Multiplex PCR**

To maximise the sensitivity of the multiplex PCR in detecting siderophore production/receptor genes, ClustalW multiple sequence alignments were performed using publically available *E. coli* sequences from EcoCyc and the National Centre for Biotechnology Information (NCBI). Primers were designed to target conserved regions of 4 biosynthetic and 1 receptor genes for all 4 siderophore systems (Table 1). Primers for the salmochelin PCR targeted export and degradation genes within the salmochelin operon as it only has 1 biosynthesis gene. Product sizes were designed to be of different lengths within one system to ensure that bands could be distinguished on a gel. Template DNA was extracted from overnight cultures using the QIAGEN DNeasy Blood and Tissue extraction kit as per manufacturer’s instructions. 10 ng of DNA was used for each multiplex PCR in a 25 µl reaction volume containing 12.5 µl Go-Taq Green Master Mix (Promega) and 0.1 mM of each primer. Amplification for each PCR was as follows: 35 cycles at

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95°C for 30s, 55°C for 30s, 72°C for 1 min, and 1 cycle at 72°C for 5 min. Yersiniabactin and aerobactin multiplex PCRs had slight alterations, with the annealing temperature raised to 60°C for the yersiniabactin PCR and elongation step shortened to 40 s for the aerobactin PCR.

RNA extraction
LB overnight cultures were washed with water and diluted to a final OD$_{600}$ of 0.05 in 250 ml flasks containing 25 ml of modified M9 medium without iron addition [14]. The cultures were incubated at 37°C in a shaking incubator until the exponential phase of growth (OD$_{600} = 0.2$). Synthesis and degradation of RNA were blocked by adding 1/5 volume of stop-solution (90% ethanol/10% phenol) [15]. The RNA was purified using the Promega SV total RNA purification kit according to the manufacturer’s instructions. Quantification of the RNA was performed by measuring the 260 nm absorbance on a Nanodrop 1000 spectrophotometer.

Real-time PCR
To determine the level of expression of each siderophore system, 5 μg of total RNA from each sample was reverse-transcribed in 20 μl of buffer containing 0.5 mM dNTPs, 0.2 μg random hexamers and 200 U reverse transcriptase (Fermentas). Target mRNAs were then detected by real-time PCR using SYBR Green JumpStart Taq ReadyMix following the manufacturer’s instructions (Sigma). Quantification was performed using the comparative Ct method relative to rpoB as an internal standard. The real-time PCR was performed using gene-specific primer pairs designed in silico (http://frodo.wi.mit.edu/) to target conserved regions (Table 1). Amplicons were designed to be 100–150 bp in size and their amplification efficiency determined to avoid potential bias. The amplification cycle was as follows: 40 cycles at 94°C for 30s, 60°C for 30s, 72°C for 40s, and 1 cycle at 72°C for 5 min.

Results
Comparison of siderophore production in E. coli populations of plant-associated and faecal isolates from healthy mammals
To assess whether environmental adaptation influences the ability of the associated E. coli populations to generate siderophores, we compared siderophore production of previously described E. coli strains either isolated from plants (GMB strains) or faecal samples of healthy mammals (ECOR-F subset; S1 Table). The isolates were grown on chrome azurol S (CAS) solid medium and the size of the halo resulting from the colour change following the accumulation of the free indicator dye was measured. Differences in growth rate were normalised by dividing the diameter of the halo by the size of the colony (Fig. 1A). No growth and no halo could be detected for 2 environmental strains (GMB37 and GMB69) and 2 faecal isolates (ECOR29 and ECOR52). Growth was not restored by adding ferrous sulphate to a 100μM final concentration to MM9, suggesting the presence of auxotrophic mutations not related to iron acquisition. These 4 strains were therefore excluded from the siderophore production comparison. Plant-associated strains produced significantly less siderophores (unpaired t-test; t = 4.39, p<0.0001) than faecal isolates on the CAS indicator agar medium (Fig. 1B). The frequency distributions of siderophore production for GMB and ECOR-F strains highlighted how a larger (37% vs 11%; Fischer’s exact test, p<0.001) proportion of ECOR-F included high siderophore producers (80th percentile) compared to GMB (Fig. 1C).

Previous epidemiological studies have shown that the iutA, iroN and fyuA siderophore receptor genes are more frequently associated with strains belonging to particular phylogenetic groups, with E. coli isolates belonging to phylogroup B2 generally possessing a greater number
Differences in siderophore production between ECOR-F and GMB could therefore be the simple reflection of the unequal phylogenetic composition of these collections [11]. However, the major E. coli phylogenetic groups were found to generate similar siderophore levels (One way ANOVA, \( p > 0.05; \text{S1 Fig.} \)), indicating that the observed differences are likely to be linked to sample origin rather than phylogenetic composition.

Design and validation of multiplex PCR for the detection of siderophore utilisation and biosynthetic genes in E. coli

To determine whether the greater capacity to produce siderophores under iron limitation displayed by the faecal isolates was also reflected at the genome level by a different complement of siderophore biosynthesis genes. We designed a multiplex PCR aimed at detecting both receptor and biosynthesis genes of the four known E. coli siderophores. We took advantage of the large number of available E. coli genome sequences to generate multiple alignments and identify suitable conserved regions to design highly sensitive and specific PCR primers (Table 1). The specificity of the multiplex PCR was assessed on the ECOR collection [12], which has been previously monitored by PCR for the presence of siderophore receptor genes [16]. More recently, the gene content of the strains from the ECOR collection has been analysed using multi-genome arrays, which cover the entire enterobactin, aerobactin and salmochelin loci [19].

We found a good correlation with the array data with 96.7% (523/541) of positive and 96.1% (518/539) of negative PCR signals matching both datasets (S2 Table). Results on the presence/absence of the yersiniabactin locus matched previously PCR-based published data in 97.2% (70/72) of cases [20].
Published PCR-based data on the distribution of siderophore receptor genes supported our results when in conflict with the array data (full details are provided in S2 Table). However, our observations that ECOR02 and ECOR67 respectively lack the aerobactin and salmochelin loci were not supported. To exclude the possibility of a sample mix up, we took advantage of existing CRISPR sequence data that can differentiate between the different ECOR strains [21]. The DNA sequences of the CRISPR2.1 and CRISPR2.3 regions of ECOR02 and ECOR67 matched those found in the databases, thus confirming the identity of the strains. We also found differences for 2 strains (ECOR11 and ECOR72) with previously published PCR-based data in the distribution of the yersiniabactin locus (S2 Table) [20] despite the fact that both CRISPR sequences confirmed the identity of these strains. The discrepancies we have identified reflect previous observations that the phenotype and genotype of ECOR strains can differ between laboratories [22], and highlight the importance of characterising the strains used in independent studies.

Distribution of the aerobactin, enterobactin, salmochelin and yersiniabactin loci in plant and faecal E. coli isolates

The distribution of the siderophore biosynthetic loci in plant-associated E. coli was assessed by performing the validated multiplex PCRs on 96 strains from the GMB collection (S3 Table) [11]. Importantly, the siderophore receptor genes were always detected alongside the corresponding biosynthetic genes (133/133, S3 Table). This observation highlights the sensitivity of our approach and indicates that each siderophore locus is generally evolutionarily maintained as a complete unit.

The prevalence of siderophore production genes observed for the strains isolated from plants was compared with that of the healthy host isolates from the ECOR-F. The results showed that, in accordance with siderophore production data, plant-associated E. coli generally possessed fewer siderophore production and uptake systems (Fig. 2). Yersinia-bactin and aerobactin were found in significantly lower proportions in plant-associated E. coli (Table 2). This is also reflected by a significant difference in the proportion of strains only possessing the enterobactin locus; 76% (73/96) and 42.6% (26/61) for GMB and ECOR-F respectively (Fischer’s exact test, p<0.0001, S2 and S3 Tables).

To expand our comparison beyond ECOR-F, assuming that the presence of the receptor genes generally correlates with the ability to synthesise the corresponding siderophores, we investigated the available recent literature for data describing the distribution of siderophore receptor genes in faecal and rectal E. coli isolates obtained from healthy humans [17,18,23–30]. Published data was consistent with data obtained for ECOR-F, confirming the narrower distribution of the aerobactin and yersiniabactin loci among the plant-associated isolates (Table 2).

Our results suggest that high siderophore production might be linked to the presence of the aerobactin and/or yersiniabactin loci. Accordingly, the presence of the aerobactin synthesis genes was nearly always associated with strong siderophore production (Table 3). However, no correlation between siderophore production and the presence of the yersiniabactin locus could be observed (Table 3). To exclude any potential effect linked to siderophore-specific differences in diffusion through the agar, the link between aerobactin and strong in vitro siderophore production was confirmed by assaying a representative subset of strains (n = 33) in liquid CAS medium (S2A Fig.). The liquid assays also reflected the CAS agar assays in showing lower siderophore production for the plant-associated strains (S2B Fig.).
Strain-specific diversity in siderophore gene expression under low iron conditions

The differential distribution of siderophore loci was not sufficient to fully explain the difference in siderophore production between GMB and ECOR-F isolates. Strains only possessing the enterobactin locus displayed a wide range of siderophore production levels, with significant differences in enterobactin production, observable between plant-associated and faecal isolates at the population level.

Fig 2. Plant associated *E. coli* isolates encode fewer siderophore production systems than faecal isolates at the population level. The graph displays the mean number of detected siderophore systems for the GMB and ECOR-F strain collections. Error bars display the standard error of the mean. Statistical significance was determined using the Student t-test. ***P < 0.001.

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the population level (unpaired t-test; \( t = 3.13, p = 0.002 \), Fig. 3). This suggested widespread differences in the regulation of siderophore production between individual \( E. coli \) strains, and thus prompted the investigation of the possible role of differences in gene regulation of enterobactin and the 3 other siderophore systems. We examined the expression levels of one biosynthesis gene for each siderophore in 8 environmental strains displaying a range of siderophore production abilities under low iron conditions. Analysis of the mRNA levels by real-time PCR showed that enterobactin and aerobactin were the most highly expressed systems, both displaying 4 and 600-fold higher expression levels than salmochelin and yersiniabactin, respectively (Fig. 4A). Importantly, enterobactin as well as salmochelin displayed a wide range of different expression levels, which strongly correlated with total siderophore production (Fig. 4B).

In contrast, aerobactin and yersiniabactin displayed a much narrower range of expression, high and very low respectively in all strains tested. This provides a possible explanation for the strong link between presence of the aerobactin locus and high siderophore production in the CAS assays (Table 3, S2A Fig.). Conversely, the generally low yersiniabactin gene expression is in good agreement with the absence of correlation between the yersiniabactin locus and siderophore production levels.

**Discussion**

Iron acquisition is vital to microbial physiology and host colonisation. Production of the enterobactin siderophore has been shown to be important for the colonisation of the healthy GI-
tract by \textit{E. coli} \textsuperscript{[31]}. However, the ability to synthesise additional siderophores is generally associated with virulence, particularly for extraintestinal pathogenic \textit{E. coli} that have reached the urinary tract or the blood stream \textsuperscript{[1,32,33]}. The observation that many commensal \textit{E. coli} isolates can produce these virulence-associated siderophores suggests another possible role in the ecology of \textit{E. coli}, perhaps including persistence outside animal hosts \textsuperscript{[4]}. Reasoning that a particular bacterial function will be more prevalent in the environment where it provides greater fitness advantage, we compared the siderophore production and distribution of siderophore biosynthetic and receptor genes in non-pathogenic \textit{E. coli} isolated from plants or mammalian hosts. This population-wide approach has previously been used to identify environment-specific differences in \textit{E. coli} carbon source utilisation and extra-cellular matrix production \textsuperscript{[11]}. We found that plant-associated isolates excrete significantly lower amounts of siderophores compared to faecal isolates when grown under iron limiting conditions. Analysis of the distribution of the 4 siderophore biosynthetic systems in both \textit{E. coli} populations showed that the aerobactin and yersiniabactin loci were less prevalent in the \textit{E. coli} strains isolated from plants. The correlation between genotype and total siderophore production suggested that the greater ability of faecal isolates to generate siderophores may be partly linked to their greater association with the aerobactin locus. Interestingly, the aerobactin biosynthesis genes are often plasmid-encoded \textsuperscript{[1,34]}. This could indicate that the lower frequency of the aerobactin locus among the GMB isolates might be linked to differences in plasmid carriage. When the comparison was restricted to strains only able to synthesise enterobactin, the difference in siderophore

\begin{figure}
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\caption{Plant associated \textit{E. coli} display lower enterobactin production compared to faecal isolates. Frequency plot comparing siderophore production of GMB and ECOR-F strains only encoding the enterobactin siderophore locus. Sample sizes in the data sets were $n_{\text{GMB}} = 69; n_{\text{ECOR-F}} = 28$.}
doi:10.1371/journal.pone.0117906.g003
\end{figure}
production was still significant between plant and faecal isolates, suggesting that diversification in the regulatory response to iron limitation may also be an important environmental adaptive trait. The possible role of differential expression of enterobactin in \textit{E. coli} adaptation mirrors previously observed differences in yersiniabactin and aerobactin synthesis between single-patient urinary and rectal \textit{E. coli} isolates [35].

Our results suggest that the environment influences the distribution of siderophore production genes among the associated \textit{E. coli} populations, and that the gut selects for \textit{E. coli} commensal strains with multiple siderophore systems and with the ability to generate higher siderophore levels under iron limitation. In contrast, the plant environment seems to exert a weaker selective pressure on \textit{E. coli} to maintain a diverse siderophore production, probably as a consequence of differences in iron source availability compared to the gut. In association with plants, \textit{E. coli} could rely on alternative iron acquisition systems such as the direct uptake of ferrichrome or ferric citrate, molecules which can be found both in rhizosphere and phyllosphere [36–38]. It would therefore be interesting to expand this type of analysis to \textit{E. coli} populations associated with other plant hosts and climates to determine whether our observations can be generalised. It is also important to note that the differences in geography and time of isolation between GMB and ECOR-F could be potential confounding factors in our study. However, geography explains only 2% of the genetic diversity between the ECOR strains isolated in Europe.
and the USA [39]. Similarly, the time of isolation has probably had little impact on the distribution of siderophore receptor genes as more recent studies of human commensal *E. coli* have reported comparable gene distribution to ECOR-F (Table 2).

Without excluding a role outside the host, production of aerobactin and yersiniabactin might therefore be important for gut colonisation by commensal *E. coli*. This hypothesis is supported by the observation that in contrast to transient *E. coli* clones, resident isolates which could be detected in the GI-tract of the same individual over 3 months or more were enriched for aerobactin biosynthesis genes [40]. Similarly, it is possible that yersiniabactin provides a fitness advantage to commensal *E. coli* since the rapid spread of the yersiniabactin locus in both virulent and commensal *E. coli* implies a role beyond pathogenesis [4,16,20].

Despite the availability of information about siderophore-mediated iron acquisition and the importance of enterobactin for *E. coli* gut colonisation [31], it is not clear how having higher and more diverse siderophore production benefits gut commensal *E. coli*. It has been shown that the 4 siderophores encoded by *E. coli* Nissle 1917 are differently affected by environmental factors such as pH and carbon source [41]. An expanded siderophore repertoire would therefore make a cell more competitive in acquiring iron in the spatially and temporally heterogeneous environment found in the gut. Strains possessing several siderophore systems could also have an advantage in the densely populated gut environment when in competition with bacteria that utilise a narrower range of siderophores.

The metabolic cost of generating siderophores is significant and has a clear impact on the *E. coli* metabolome [42]. Indeed, it has been shown that under specific circumstances bacteria can lose the ability to make siderophores while retaining the machinery for acquiring those produced by other strains, thus opening the possibility of cheating [43,44]. However, we observed that the siderophore receptor genes were always detected alongside the corresponding biosynthetic genes. This suggests that the natural environment selects against *E. coli* cheaters. One possible explanation might be the poor diffusion of siderophores in viscous media such as mucus [45], where *E. coli* is thought to reside, making it less likely for a cheater to be in contact with significant amounts of the required siderophore. This hypothesis is supported by the observation that enterobactin hyperexcretion makes catecholate transport-deficient *E. coli* iron-starved *in vivo* despite retaining the ability to import ferric iron through other systems [31]. This phenomenon could be explained by the entrapment of enterobactin around mucus-embedded cells, thus making the iron inaccessible to other acquisition systems.

In summary, our data suggest that the ability to produce yersiniabactin and aerobactin is associated with the colonisation of the gut by *E. coli*. The conditions under which each siderophore system is expressed and influences *E. coli* fitness in the GI-tract requires further investigation, as does the extent to which our findings can be extended to the persistence of pathogenic *E. coli*, such as those strains causing infections of the urinary tract, for which the physiologically normal gut is a likely reservoir [46,47].

**Supporting Information**

**S1 Fig. Phylogenetic distribution of siderophore production.** Box plots showing siderophore production by GMB and ECOR-F strains split according to the major *E. coli* phylogenetic groups. The central rectangle of the plot spans the interquartile range (IQR). The segment inside the rectangle shows the median, while the whiskers span the 5–95 percentile. Black circles represent outliers. Statistical significance was determined using the Student *t*-test. No significant differences were detected.

(PDF)
S2 Fig. Siderophore production of GMB and ECOR-F strains as assessed by liquid CAS assay. Box plots showing siderophore production in liquid CAS for a subset of strains (n = 33) to visualise differences in siderophore production of A) strains with or without the aerobactin biosynthesis locus, and B) GMB and ECOR-F. The central rectangle of the plot spans the inter-quartile range (IQR). The segment inside the rectangle shows the median, while the whiskers span the 5–95 percentile. Black circles represent outliers. Statistical significance was determined using the Student t-test; *P<0.05, ****P<0.0001.

S1 Table. Siderophore production of GMB and ECOR-F strains as assessed by CAS agar assay. The table displays the ratios between colony and halo diameter as measured on chrome azurol S agar plates for each GMB and ECOR-F strain.

S2 Table. Distribution of enterobactin, aerobactin, yersiniabactin and salmochelin loci in the ECOR collection as assessed by multiplex PCR. The table shows presence/absence of the genes encoded in siderophore production loci in E. coli isolates from the ECOR strain collection.

S3 Table. Distribution of enterobactin, aerobactin, yersiniabactin and salmochelin loci in the GMB collection as assessed by multiplex PCR. The table shows presence/absence of the genes encoded in siderophore production loci in E. coli isolates from the GMB strain collection.

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Author Contributions
Conceived and designed the experiments: LJS GM SL. Performed the experiments: LJS GM. Analyzed the data: LJS GM IP SL. Contributed reagents/materials/analysis tools: SKS. Wrote the paper: LJS GM SL.

References


