

## ORIGINAL ARTICLE

# Functional metagenomics reveals novel salt tolerance loci from the human gut microbiome

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**Metagenomics is a powerful tool that allows for the culture-independent analysis of complex microbial communities. One of the most complex and dense microbial ecosystems known is that of the human distal colon, with cell densities reaching up to  $10^{12}$  per gram of faeces. With the majority of species as yet uncultured, there are an enormous number of novel genes awaiting discovery. In the current study, we conducted a functional screen of a metagenomic library of the human gut microbiota for potential salt-tolerant clones. Using transposon mutagenesis, three genes were identified from a single clone exhibiting high levels of identity to a species from the genus *Collinsella* (closest relative being *Collinsella aerofaciens*) (COLAER\_01955, COLAER\_01957 and COLAER\_01981), a high G + C, Gram-positive member of the Actinobacteria commonly found in the human gut. The encoded proteins exhibit a strong similarity to GalE, MurB and MazG. Furthermore, pyrosequencing and bioinformatic analysis of two additional fosmid clones revealed the presence of an additional *galE* and *mazG* gene, with the highest level of genetic identity to *Akkermansia muciniphila* and *Eggerthella* sp. YY7918, respectively. Cloning and heterologous expression of the genes in the osmosensitive strain, *Escherichia coli* MKH13, resulted in increased salt tolerance of the transformed cells. It is hoped that the identification of atypical salt tolerance genes will help to further elucidate novel salt tolerance mechanisms, and will assist our increased understanding how resident bacteria cope with the osmolarity of the gastrointestinal tract.**

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## Introduction

The ability to respond and adapt to changes in external osmolarity is a key determinant for bacterial survival and proliferation in various environmental niches (Sleator and Hill, 2002). Microorganisms are continually exposed to fluctuations and perturbations in osmolarity in their environment caused by rainwater, drought, salinity and changing solute concentrations. Both transient and symbiotic microorganisms that colonize the gastrointestinal tract are particularly susceptible to water loss due to osmotic stress (Gralla and Huo, 2008). It has been demonstrated that free water is not evenly distributed along the gut, but exists as pockets, meaning changes in osmolarity can be rapid (Schiller *et al.*, 2005). The elevated osmolarity of the upper small intestine

(the equivalent of 0.3 M NaCl (Chowdhury *et al.*, 1996)), represents an initial challenge to ingested microorganisms and the osmolarity is likely to be higher in the distal colon following further water absorption in the final stages of the digestive process. *Bacteroides fragilis* isolates from human stool samples have shown increased resistance to both NaCl and bile stress compared with isolates from blood or abscesses, indicating the potential importance of such stress tolerance mechanisms in the gastrointestinal tract (Pumbwe *et al.*, 2007).

In general, bacteria respond to hyper-osmotic stress in a phased manner. Firstly, during the primary response, which is activated within seconds of osmotic upshift, potassium ( $K^+$ ) is rapidly transported into the cell (for a review see Epstein, 2003). However, a more sophisticated osmotic stress response system is required once the cellular osmolality has been initially stabilized. The next phase, or secondary response, involves the uptake and/or synthesis of compatible solutes (also termed osmolytes or osmoprotectants). As their name suggests, these diverse compounds (Kempf and Bremer, 1998) are for the most part compatible with

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vital cellular functions and can restore cell volume and turgor without adversely affecting metabolism (Kunte, 2006). Although the primary and secondary responses constitute the classical response to hyperosmotic stress, the overall osmoadaptation strategy is much more complex, and involves a diverse range of cellular systems and processes seemingly unrelated to the primary and secondary responses. Identifying such diverse genes/proteins will provide us with a broader and more complete view of salt tolerance and the cellular response to salt-induced osmotic stress in bacteria. Indeed, many such ancillary mechanisms have been identified and range from two-component systems and proteolytic systems to numerous general stress and heat stress proteins and can also involve changes to the cell membrane (Kilstrup *et al.*, 1997; Sakamoto and Murata, 2002; Gardan *et al.*, 2003; Piuri *et al.*, 2003; Wonderling *et al.*, 2004; Sleator and Hill, 2005; Lopez *et al.*, 2006).

Since their inception, metagenomic strategies have led to the identification of numerous novel and diverse genes, enzymes and proteins from many diverse environments through sequence-based and/or functional approaches (Beja *et al.*, 2000; Gillespie *et al.*, 2002; Lee *et al.*, 2007; Banik and Brady, 2008; Heath *et al.*, 2009; Meilleur *et al.*, 2009). In principle, metagenomics can provide access to all of the genetic resources in a given environmental niche and as such is an extremely powerful tool to access the genomes of difficult-to-culture or unculturable microorganisms (Sleator *et al.*, 2008). A recent study identified novel genes from a pond water metagenome that increased resistance to salt-induced osmotic stress when expressed in *E. coli* (Kapardar *et al.*, 2010a, b). Although some studies have used functional metagenomics to screen for various

phenotypes from the human gut environment (Kazmierczak *et al.*, 2008; Lakhdari *et al.*, 2010; Gloux *et al.*, 2011), to our knowledge this study is the first to identify genes that confer salt tolerance from the human gut microbiota.

## Materials and methods

### Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Primers (Eurofins, MWG Operon, Ebersberg, Germany) used in this study are listed in Table 2. *E. coli* EPI300::pCC1FOS (Epicentre Biotechnologies, Madison, WI, USA) was grown in Luria–Bertani (LB) medium (Maniatis *et al.*, 1982) containing 12.5 µg ml<sup>-1</sup> chloramphenicol (Cm) and in 12.5 µg ml<sup>-1</sup> chloramphenicol plus 50 µg ml<sup>-1</sup>

**Table 2** Primers used in this study

Primer	Sequence (5'–3') <sup>a</sup>
pCI372 FP	CGGGAAGCTAGAGTAACTAG
pCI372 RP	CCTCTCGGTTATGAGTTAG
<i>mazG</i> (3) FP	AAA <u>AACTGCAGG</u> CCCGTCGTTCCCGCAGTCTTAC
<i>mazG</i> (3) RP	GCTCTAG <u>AATCTACG</u> AGGGCGCGCGGTTTC
<i>murB</i> (3) FP	AAA <u>AACTGCAG</u> CCACCTCCTGGGGGATCTGCTTGAG
<i>murB</i> (3) RP	GCTCTAG <u>ACGACACAC</u> CGGACTGGGTTATCTGA
<i>galE</i> (3) FP	AAA <u>AACTGCAG</u> ATGGGTGTGCAGTCCGCCTC
<i>galE</i> (3) RP	GCTCTAG <u>AGTCCCAACG</u> ATTTCACGAAACG
<i>mazG</i> (5) FP	AAA <u>AACTGCAG</u> CTAAACAGGAGGCGAAGCTC
<i>mazG</i> (5) RP	GCTCTAG <u>AGCAGAAG</u> CGCTCAACGATA
<i>galE</i> (25) FP	GCTCTAG <u>ACCGGCTTAA</u> CAGCATTGATA
<i>galE</i> (25) RP	AAA <u>AACTGCAGG</u> CTGCGTGTCTTTCCAGTT
EZTn-FP-1	GCCAACGACTACGCACTAGCCAAC
EZTn-RP-1	GAGCCAATATGCGAGAACACCCGAGAA
T7	TAATACGACTCACTATAGGG
M13 R	CAGGAAACAGCTATGACC

Abbreviations: FP, forward primer; RP, reverse primer.

<sup>a</sup>Restriction enzyme recognition sequences are underlined.

**Table 1** Bacterial strains, plasmids and transposon used in this study

Strain, plasmid or transposon	Genotype or characteristic(s)	Source or reference
<b>Strains</b>		
<i>E. coli</i> EPI300	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) Φ80 <i>dlacZAM15</i> Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ( <i>ara, leu</i> )7697 <i>galU</i> <i>galK</i> λ <i>rpsL</i> <i>nupG</i> <i>trfA</i> <i>dhfr</i> ; high-transformation efficiency of large DNA	Epicentre Biotechnologies
<i>E. coli</i> MKH13	MC4100A( <i>putPA</i> )101D( <i>proP</i> )2D( <i>proU</i> )	(Haardt <i>et al.</i> , 1995)
<i>E. coli</i> MKH13::pCI372- <i>murB</i> (3)	MKH13 containing pCI372 with <i>murB</i> gene from SMG 3 (similar to <i>Collinsella aerofaciens</i> ATCC 25986)	This study
<i>E. coli</i> MKH13::pCI372- <i>mazG</i> (3)	MKH13 containing pCI372 with <i>mazG</i> gene from SMG 3 (similar to <i>Collinsella aerofaciens</i> ATCC 25986)	This study
<i>E. coli</i> MKH13::pCI372- <i>galE</i> (3)	MKH13 containing pCI372 with <i>galE</i> gene from SMG 3 (similar to <i>Collinsella aerofaciens</i> ATCC 25986)	This study
<i>E. coli</i> MKH13::pCI372- <i>mazG</i> (5)	MKH13 containing pCI372 with <i>mazG</i> gene from SMG 5 (similar to <i>Eggerthella</i> sp. YY7918)	This study
<i>E. coli</i> MKH13::pCI372- <i>galE</i> (25)	MKH13 containing pCI372 with <i>galE</i> gene from SMG 25 (similar to <i>Akkermansia muciniphila</i> ATCC BAA-835)	This study
<b>Plasmids</b>		
pCI372	Shuttle vector between <i>E. coli</i> and <i>L. lactis</i> , Cm <sup>R</sup>	(Hayes <i>et al.</i> , 1990)
pCC1FOS	Fosmid cloning vector, Cm <sup>R</sup>	Epicentre Biotechnologies
<b>Transposon</b>		
EZ-Tn5 < <i>oriV</i> /KAN-2>	Hyperactive Tn5 transposon, Kan <sup>R</sup> , inducible high copy origin of replication— <i>oriV</i>	Epicentre Biotechnologies

Kan (kanamycin) following EZ-Tn5 transposon mutagenesis reactions. *E. coli* MKH13 was grown in LB medium and in LB medium supplemented with  $20 \mu\text{g ml}^{-1}$  Cm for strains transformed with the plasmid pCI372. LB media was supplemented with 1.5% agar when required. All overnight cultures were grown at  $37^\circ\text{C}$  with shaking.

#### *Metagenomic library construction*

A metagenomic library that had been constructed previously (Jones and Marchesi, 2007) was used to screen for salt-tolerant clones. Briefly, metagenomic DNA was isolated from a human faecal sample and from this a fosmid library (average insert size of approximately 40 kb) was created using a Copy Control fosmid library production kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. A Genetix (Berkshire, UK) QPix 2 XT colony-picking robot was used to transfer fosmid clones to 384-well micro-titre plates, which were stored at  $-80^\circ\text{C}$  until needed.

#### *Screening the metagenomic library*

A total of 23 040 clones from the library (average insert size of  $\sim 40$  kb) were screened on LB agar supplemented with 6.5% NaCl for clones showing increased tolerance to NaCl compared with the *E. coli* EPI300 host strain (containing an empty pCC1FOS cloning vector). A Genetix QPix 2 XT colony picking/gridding machine was used to plate the clones onto Q-Trays (Genetix) containing LB agar supplemented with  $12.5 \mu\text{g ml}^{-1}$  Cm and 6.5% NaCl. Q-Trays were incubated at  $37^\circ\text{C}$  for 72 h. Following incubation, likely salt-tolerant clones were replica plated onto LB containing  $12.5 \mu\text{g ml}^{-1}$  Cm and 6.5% NaCl and onto LB containing  $12.5 \mu\text{g ml}^{-1}$  but without NaCl (which served as a positive control).

#### *Growth experiments*

Cultures were grown overnight with shaking in LB broth. Subsequently, cells were harvested, washed in one quarter strength sterile Ringer's solution and resuspended in fresh LB broth. A 2% inoculum was sub-cultured in fresh LB broth containing the appropriate stress (that is, 0–10% (w/v) NaCl, 0–8% (w/v) KCl, 0–90% (w/v) sucrose or 0–80% (v/v) glycerol as required) and 200  $\mu\text{l}$  was transferred to a sterile 96-well micro-titre plate (Starstedt Inc. Newton, MA, USA). Plates were incubated at  $37^\circ\text{C}$  for 48 h in an automated spectrophotometer (Tecan Genios, Mannedorf, Switzerland), which recorded the optical density 595 nm every hour. After 48 h, the data was retrieved and analysed using the Magellan 3 software program. Results are presented as the average of triplicate experiments, with error bars being representative of the s.e.m.

#### *DNA manipulations*

Extraction of fosmids containing metagenomic DNA: 5 ml of bacterial culture was grown overnight with  $12.5 \mu\text{g ml}^{-1}$  Cm. To inoculate 4 ml of fresh LB broth, 1 ml of culture was used. To this, 5  $\mu\text{l}$  of 1000  $\times$  Copy Control Induction solution (Epicentre Biotechnologies) and  $12.5 \mu\text{g ml}^{-1}$  Cm were added. The mixture was incubated at  $37^\circ\text{C}$  for 5 h with vigorous shaking (200–250 r.p.m.) to ensure maximum aeration. Cells were harvested from the whole 5 ml of induced culture by centrifuging at  $2100 \times g$  for 12 mins. Qiagen (West Sussex, UK) QIAprep Spin mini-prep kit was used to extract fosmids as per manufacturer's instructions. PCR products were purified with a Qiagen PCR purification kit and digested with *Xba*I and *Pst*I (Roche Applied Science, West Sussex, UK), followed by ligation (T4 DNA ligase (Roche Applied Science) for *mazG*(3) and *murB*(3); FastLink DNA ligase for *galE*(3), *galE*(25) and *mazG*(5) (Epicentre Biotechnologies) as per manufacturer's instructions) to similarly digested plasmid pCI372. Electrocompetent *E. coli* MKH13 were transformed with the ligation mixture and plated on LB agar plates containing  $20 \mu\text{g ml}^{-1}$  Cm for selection. Colony PCR was performed on all resistant transformants using primers across the multiple cloning site (MCS) of pCI372 to confirm the presence and size of the insert.

#### *Transposon mutagenesis*

Transposon mutagenesis was carried out on SMG 3 according to the manufacturer's instructions using the EZTn-5 <oriV/ KAN-2> *in vitro* transposition kit (Epicentre Biotechnologies). *E. coli* EPI300 cells were transformed with the transposon reaction mixture and selected on plates containing Cm and Kan ( $12.5$  and  $50 \mu\text{g ml}^{-1}$ , respectively). Subsequently, the transposon insertion clones were replica plated onto LB with and without 6.5% NaCl. Clones which grew on LB but not on LB + 6.5% NaCl indicates a likely insertion event in a gene involved in salt tolerance. Presumptive salt-tolerant knockouts were grown overnight and a fosmid DNA extraction was performed. The extracted fosmid containing metagenomic DNA was subjected to sequencing from the ends of the transposon using the primers EZTn-FP-1 and EZTn-RP-1 (Table 2). All sequencing was performed by GATC Biotech (Konstanz, Germany).

#### *Sequencing and bioinformatic analysis*

Fosmids SMG 1, 3, 5, 6 and 25 were fully sequenced and assembled by GATC Biotech using the GS FLX (Roche Applied Science) platform. Putative open reading frames were predicted using Softberry FGENESB bacterial operon and gene prediction software (Mavromatis *et al.*, 2007). Retrieved nucleotide and translated amino-acid sequences

were functionally annotated by homology searches using the Basic Local Alignment and Search Tool (BLAST), to identify homologous sequences from the National Centre for Biotechnology Information website: <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>.

End sequencing was performed on all fosmid clones (SMG1-53) using T7 or M13 R primers and the taxonomic distribution of end sequences were assigned using the BLAST program (Supplementary Table S1).

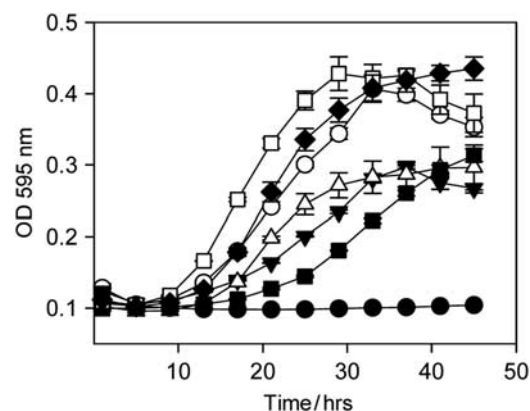
Nucleotide sequences identified in this study were compared against the healthy individuals in the MetaHit data set (Qin *et al.*, 2010) using the BLAST program to identify homologous sequences (>50% identity;  $e$ -value <  $1 \times 10^{-5}$ ). This data was used to determine the relative abundance of the identified genes in the data set and estimate the amount of DNA that would need to be screened (Mb) to return a hit to one of the genes (Supplementary Figures S2A–C).

In addition, BLASTP analysis was undertaken to identify the protein sequences in GenBank, which showed the highest identity to genes isolated here and which were shown to be responsible for the salt tolerance phenotype. The closest hits were aligned using ClustalW (Thompson *et al.*, 1994) in Bioedit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The aligned proteins were analysed using MEGA 5 (Tamura *et al.*, 2011) and the evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987) from the evolutionary distances, which were computed using the Poisson correction method (Zuckerkanndl and Pauling, 1965) and are in the units of the number of amino-acid substitutions per site. The bootstrap method (500 replicates) was used to test the percentage of replicate trees in which the associated taxa clustered together (Felsenstein, 1985) (Supplementary Figure S3).

## Results

### Screening the metagenomic library

In this study, a metagenomic fosmid library constructed from DNA isolated from human gut microbiota was screened for clones, which conferred increased salt tolerance using a combined functional metagenomic, transposon mutagenesis and bioinformatic analysis approach. The cloning host is incapable of growth on LB supplemented with 6.5% NaCl, providing a positive selection for clones able to cope with elevated osmolarity. In total, 53 salt-tolerant clones were identified, which were annotated SMG (salt metagenome) 1–53. Six clones (SMG 1–6) grew within 24 h; while a further 47 CFUs appeared within 36 h. Physiological growth experiments were conducted on host strain EPI300::pCC1FOS and SMG 1–6. All six clones showed increased salt tolerance relative to the control at 7% NaCl (*E. coli* EPI300::pCC1FOS host strain) (Figure 1). The clones were subjected to further study through transposon mutagenesis.



**Figure 1** Growth in LB broth supplemented with 7% NaCl. Growth of *E. coli* EPI300::pCC1FOS host strain (●), SMG 1 (○), SMG 2 (▼), SMG 3 (△), SMG 4 (■), SMG 5 (□) and SMG 6 (◆).

### Transposon mutagenesis

Transposon mutagenesis was carried out using the EZ-Tn5 <oriV/ KAN-2> insertion kit (Epicentre Biotechnologies). Transposon mutagenesis proved unsuccessful for SMG 1, SMG 5 and SMG 6, possibly owing to difficulty in obtaining sufficiently high yields of fosmid DNA for *in vitro* transposon mutagenesis, so SMG 3 was chosen for further investigation. Transposon mutagenesis of SMG 3 yielded nine clones incapable of growth on 6.5% NaCl. Sequencing revealed transposon insertions in three distinct genes with high genetic identity (95–98%) to genes encoding hypothetical proteins from a member of the genus *Collinsella* (closest relative being *C. aerofaciens*). The genes; *galE*(3) (COLAER\_01955), *murB*(3) (COLAER\_01957) and *mazG*(3) (COLAER\_01981), encode hypothetical proteins similar to UDP-glucose 4-epimerase (GalE), UDP-N-acetylenolpyruvoylglucosamine reductase (MurB) and nucleoside triphosphate pyrophosphohydrolase (MazG family protein). Although GalE has been tentatively linked to salt tolerance in previous studies (Hengge-Aronis *et al.*, 1991; Bohringer *et al.*, 1995; Nguyen *et al.*, 2004) we are unaware of any previous link between MurB or MazG and bacterial salt tolerance.

### Sequencing and bioinformatic analysis

End sequencing was carried out on all 53 SMG clones, using T7 or M13 R primers (GATC Biotech). In all, 49 clones were successfully end sequenced and taxonomically assigned based on BLAST hits. Of these, the *Bacteroidetes* dominated, representing 57.14% of the sequences, followed by the *Actinobacteria* and *Proteobacteria* at 14.29% and 12.24%, respectively. The *Verrucomicrobia* made up 8.16% of the sequences, whereas the *Firmicutes* were under-represented at 4.08%. The remainder of the sequences were unidentified and one sequence came from a member of the *Heterokontophyta*



(*Blastocystis hominis*), a eukaryote (2.04% each). (Supplementary Table S1, Supplementary Material).

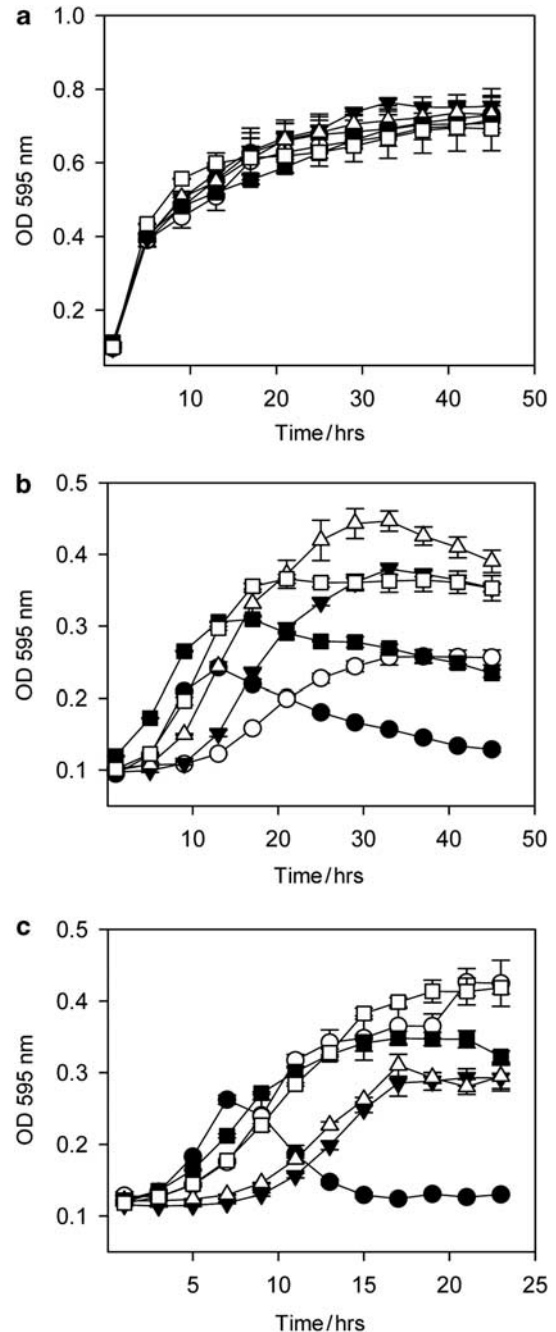
Full fosmid sequencing was performed on clones SMG 1, 3, 5, 6 and 25, revealing insert sizes of 36, 39, 42, 36 and 44 kb, respectively. The sequences have been submitted to GenBank; with following accession numbers: SMG 1=JQ269596; SMG 3=JQ269597; SMG 5=JQ269598; SMG 6=JQ269599; SMG 25=JQ269600; SMG 1 and SMG 6 were found to have the same metagenomic insert, with the highest genetic identity to *Bacteroides thetaiotaomicron*. SMG 5 shared highest genetic identity to *Eggerthella* sp. YY7918 (a member of the high G+C Gram-positive *Actinobacteria*). However, it should be noted that BlastP analysis of the predicted proteins encoded by the SMG 5 genes revealed many variations in the associated species. While all but one corresponded to members of the phylum *Actinobacteria*, the genera were represented by various species of *Eggerthella*, *Slackia*, *Cryptobacterium* and *Gordonibacter*. This indicates that the SMG 5 insert represents DNA from a novel species from one of these genera. SMG 25 shared highest genetic identity to *Akkermansia muciniphila* (a member of the Gram-negative *Verrucomicrobia*). Putative open reading frames were identified using FGENESB bacterial operon and gene prediction software from Softberry (Mavromatis *et al.*, 2007). BLAST analysis revealed the presence of a hypothetical protein (EGYY\_03530) and a UDP glucose 4-epimerase encoded by putative *mazG* and *galE* genes, respectively. These genes were present on SMG 5 and SMG 25 and were designated *mazG*(5) and *galE*(25), respectively. Having previously cloned and expressed homologous genes (*galE*(3) and *mazG*(3)) from SMG 3 in *E. coli* MKH13, resulting in an increased salt tolerance phenotype, it was decided to clone these bioinformatically identified *mazG*(5) and *galE*(25) genes into *E. coli* MKH13 also.

The nucleotide sequences of the genes identified were compared with the MetaHit data set from the healthy individuals (Qin *et al.*, 2010) to determine the relative abundance of the genes among the gut metagenomes of the subjects. Homologues of all of the genes identified in this study were found to be present in all MetaHit samples (>50% identity; *e*-value <1 × 10<sup>-5</sup>). The *galE* genes (*galE*(3) and *galE*(25)) were found to be much more abundant than both *mazG*(3) and *murB*(3), with a hit rate of approximately 1 per Mb of DNA screened, compared with approximately one per 4–6 Mb DNA for *mazG* and *murB* (Supplementary Figures S2A–C).

#### Growth experiments

Three genes were identified by transposon mutagenesis from SMG 3, namely *galE*(3), *murB*(3) and *mazG*(3) and two further genes were identified through bioinformatic analysis; *mazG*(5) from SMG 5 and *galE*(25) from SMG 25. The genes were cloned

with some flanking DNA into the shuttle plasmid pCI372 and transformed into electrocompetent *E. coli* MKH13. Growth experiments in LB and LB supplemented with NaCl or KCl (ionic osmotic stress) were conducted on MKH13::pCI372-*mazG*(3),



**Figure 2** Growth in LB and LB supplemented with 3% NaCl or 4% KCl. The first *P* value in parentheses represents growth in NaCl, while the second *P* value represents growth in KCl. Growth of WT MKH13 (●), MKH13::pCI372-*galE*(3) (*P* < 0.006; *P* = 0.0004) (○), MKH13::pCI372-*murB*(3) (*P* < 0.0001; *P* < 0.0001) (△), MKH13::pCI372-*mazG*(3) (*P* < 0.0001; *P* < 0.0017) (▼), MKH13::pCI372-*galE*(25) (*P* = 0.0002; *P* = 0.0012) (■) and MKH13::pCI372-*mazG*(5) (*P* = 0.0003; *P* < 0.0001) (□) in (a) LB broth, (b) LB broth supplemented with 3% NaCl and (c) LB broth supplemented with 4% KCl.

MKH13::pCI372-*murB*(3), MKH13::pCI372-*galE*(3), MKH13::pCI372-*mazG*(5) and MKH13::pCI372-*galE*(25). Each of the five transformed clones showed a statistically significant increase in salt tolerance (to both NaCl and KCl) compared with wild-type MKH13 (Figures 2b and c), whereas no difference in growth was observed in LB alone (Figure 2a). Each of the five transformed clones was also tested in their ability to grow under conditions of non-ionic osmotic stress (that is, sucrose or glycerol). No increased growth phenotype was observed under these conditions for any of the clones (Supplementary Figure S1), indicating these genes may confer a salt-specific protective effect.

## Discussion

Metagenomics has the potential to allow us to advance, or for the most part to begin, the study of the genetic complement of uncultured microbes. In the current study, we used a combined functional metagenomic, transposon mutagenesis and bioinformatic strategy to screen a metagenomic library from the human gut microbiota for potential salt-tolerant clones and identified five genes (Table 3), namely *galE*(3) and *galE*(25), *mazG*(3) and *mazG*(5) and *murB*(3), involved in salt tolerance and likely to be important for life in the gut.

In the most comprehensive analysis to date of the genetic complement of the human gut microbiome, a cohort of over 1200 genes (termed range clusters) were identified, which encode functions important for life in the gut (Qin *et al.*, 2010). These included genes, which encoded proteins similar to those that we have identified in this study, namely, UDP glucose 4-epimerase, NTP pyrophosphohydrolase (for which MazG is a functional homologue), as well as a protein containing a tetrapyrrole methyltransferase domain and a MazG-like domain and UDP-*N*-acetylmuramate dehydrogenase (which is

another name for MurB; UDP-*N*-acetylenolpyruvylglucosamine reductase) (Supplementary Table S10 from Qin *et al.*, 2010). The presence of the genes encoding these proteins in the enriched gene set leads us to conclude that they are important for survival in the gut and their putative role in adapting to fluctuating levels of osmotic stress in the gut. Also, homologues of the identified sequences from this study were found to be abundant in each of the individual metagenomes from the MetaHit data set (Supplementary Figures S2A–C). Furthermore, an analysis of human gut genomic and metagenomic data sets identified uncharacterized and novel protein families, which are over-represented in the human gut (Ellrott *et al.*, 2010). Among these, and second on the list of over-represented protein families, are coiled-coil osmosensory transporters. The best characterized of the coiled-coil transporters is ProP of *E. coli*, which is responsible for the uptake of osmoprotectants, such as proline and betaine during osmotic stress (Culham *et al.*, 1993). Such representative proteins were not found in any of the genomes analysed from microbes not associated with the human gut microbiome, indicating their importance to bacteria residing in the human gut. This observation also reinforces the idea that the human gut microbiota may employ novel strategies and possess novel mechanisms for osmoadaptation.

Transposon mutagenesis resulted in the identification of a gene (*galE*) from SMG 3 (*galE*(3)), encoding a UDP glucose 4-epimerase (GalE) (also termed UDP galactose 4-epimerase), whereas another *galE* gene was bioinformatically identified from SMG 25 (*galE*(25)). This enzyme catalyses the direct inter-conversion of UDP glucose and UDP galactose (Leloir, 1951), which are precursors involved in the synthesis of capsular polysaccharide and the compatible solute trehalose as well as the synthesis of lipopolysaccharide and membrane-derived oligosaccharides in Gram-negative bacteria

**Table 3** Genes identified in this study

SMG clone	Putative gene	Method of identification	Top BLAST hit	% Identity (amino acid)	e-value
SMG 3–3	<i>mazG</i> (3) COLAER_01981 (861 bp)	EZ-Tn5 transposon mutagenesis	Hypothetical protein; <i>Collinsella aerofaciens</i> , Similar to <i>mazG</i> encoding a MazG family protein (nucleoside triphosphate pyrophosphohydrolase) (286 aa)	95	2e-94
SMG 3–11	<i>murB</i> (3) COLAER_01957 (963 bp)	EZ-Tn5 transposon mutagenesis	Hypothetical protein; <i>Collinsella aerofaciens</i> , Similar to <i>murB</i> encoding UDP- <i>N</i> -acetylenolpyruvoylglucosamine reductase (320 aa)	98	5e-83
SMG 3-17	<i>galE</i> (3) COLAER_01955 (1062 bp)	EZ-Tn5 transposon mutagenesis	Hypothetical protein; <i>Collinsella aerofaciens</i> , Similar to <i>galE</i> encoding UDP glucose 4-epimerase (353 aa)	98	3e-175
SMG 5	<i>mazG</i> (5) EGYY_03530 (876 bp)	Bioinformatic analysis	Hypothetical protein; <i>Eggerthella</i> sp. YY7918, Similar to <i>mazG</i> encoding a MazG family protein (nucleoside triphosphate pyrophosphohydrolase) (291 aa)	61	1e-117
SMG 25	<i>galE</i> (25) Amuc_1125 (990 bp)	Bioinformatic analysis	Hypothetical protein; <i>Akkermansia muciniphila</i> , Similar to <i>galE</i> encoding a UDP glucose 4-epimerase (329 aa)	96	0.0

and lipoteichoic acids in Gram-positive bacteria (Fukasawa *et al.*, 1962; Markovitz, 1977; Schulman and Kennedy, 1977; Giaever *et al.*, 1988; Seltman and Holst, 2002; Grundling and Schneewind, 2007).

The *galE* gene is often found in an operon with the *galT* and *galK* genes for galactose metabolism. In SMG 3 (highest genetic identity to *C. aerofaciens*), the *galE* gene (COLAER\_01955) is found not on a *gal* operon, but between two genes encoding hypothetical proteins similar to a peptidase and a 4- $\alpha$ -glucanotransferase (COLAER\_01956 and COLAER\_01953, respectively). COLAER\_01957 (*murB*) is located 226 nucleotides downstream of COLAER\_01956. *C. aerofaciens'* *galE* may still be involved in galactose metabolism, as the organism can utilize galactose (Kageyama *et al.*, 1999). The *galE* gene from SMG 25 is not found on the galactose operon and also exhibits a different genomic arrangement to SMG 3. This gene, *galE*(25), which shares 96% identity to *A. muciniphila* (Amuc\_1125), is located between a UDP-galactopyranose mutase homologue from *Chthoniobacter flavus* (52% identity) and a gene encoding a hypothetical protein similar to Amuc\_1123 from *A. muciniphila*. Furthermore, the fosmid insert of SMG 25 has a vastly different genomic arrangement to that of *A. muciniphila*. This lack of synteny indicates that the fosmid insert is not from *A. muciniphila*, but from a related but as yet undiscovered species. Indeed, sequences representing at least eight uncharacterized species of the genus *Akkermansia* have been identified from different gut metagenomic libraries (van Passel *et al.*, 2011). Interestingly, a UDP glucose 4-epimerase homologue has been identified as a putative gene involved in drought tolerance in rice by modulating the ability of rice roots to penetrate deeper into the substratum when exposed to drought conditions (Nguyen *et al.*, 2004).

A transposon insertion in the *galE* could potentially affect the cell in different ways; (1) by causing compositional changes in the lipopolysaccharide layer or in lipoteichoic acids of Gram-negative and Gram-positive bacteria, respectively, thus reducing the cell's ability to sense, respond to or resist various environmental stress conditions and (2) by disrupting the inter-conversion of UDP-glucose and UDP-galactose. UDP glucose may be a key molecule, as it can be converted to the osmoprotectant trehalose (Giaever *et al.*, 1988), in addition to potentially modulating the expression of RpoS (which increases expression of a number of genes at high salt concentrations (Hengge-Aronis *et al.*, 1991; Bohringer *et al.*, 1995)).

Expressing this *galE* gene in *E. coli* may result in increased UDP glucose levels, which could be converted to trehalose resulting in an osmoprotective effect. Padilla *et al.* (2004) increased the UDP glucose supply and consequently the levels of accumulated trehalose by expressing the *E. coli galU* gene in *Corynebacterium glutamicum*. In our study, the expression of both the *galE*(3) and *galE*(25) genes

in the osmosensitive strain *E. coli* MKH13 resulted in a statistically significant increased salt (NaCl and KCl) tolerance phenotype (Figures 2b and c). Of the five genes identified and cloned, *galE*(3) had a lesser effect than the other four genes. MKH13::pCI372-*galE*(3) also had a prolonged lag phase. It has been demonstrated that cellular stress may be caused by imbalances and accumulation of certain intermediary metabolites, particularly in the case of amphibolic pathways (for example, D-galactose pathway), as demonstrated in a *galE* mutant (Lee *et al.*, 2009). In the current study, supplying MKH13 with an additional plasmid-encoded copy of *galE* may also cause an imbalance in an intermediary metabolite, leading to stress and ultimately a prolonged lag phase, which ends when the imbalance is corrected and homeostasis is restored.

Peptidoglycan is a major component of the bacterial cell wall and has an important role in withstanding osmotic stress (van Heijenoort, 1996). MurB is essential for cell wall biosynthesis and is involved in a two-step process with UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) to form UDP-N-acetylmuramate, which is a building block for peptidoglycan (Sylvester *et al.*, 2001). The *murB* gene has been shown to be essential for normal growth in *Bacillus subtilis*, *E. coli* and *Staphylococcus aureus* (Miyakawa *et al.*, 1972; Matsuo *et al.*, 2003; Real and Henriques, 2006). In *S. aureus*, a mutation within the *murB* gene resulted in thermosensitive mutants, which had thinner cell walls (Matsuo *et al.*, 2003). The *murB*(3) gene conferred an increased growth phenotype to *E. coli* MKH13 during both NaCl and KCl stress (Figures 2b and c). Disruption or deletion of the *murB* gene could make cells acutely sensitive to osmotic stress owing to a reduction in cell wall integrity as well as causing a reduction in turgor pressure, which is a driving force for cellular growth and division. Bacteria remodel the structure of their peptidoglycan in response to changes in environmental conditions (Vijaranakul *et al.*, 1995; Quintela *et al.*, 1997), which could be important in the gut by allowing for varying levels of rigidity or elasticity depending on the conditions in the immediate environment.

We have also identified two putative MazG family proteins (encoded by the *mazG* gene from SMG 3 and SMG 5 (*mazG*(3) and *mazG*(5), respectively). These are highly conserved proteins in bacteria and to date there has been no tangible link demonstrated between MazG and salt tolerance. MazG is a nucleoside triphosphate pyrophosphohydrolase (NTPase), which can hydrolyse (deoxy)ribonucleoside triphosphates ((d)NTPs) to their corresponding (deoxy)ribonucleoside monophosphates ((d)NMPs) and pyrophosphate (PPi) (Zhang and Inouye, 2002). It has been proposed that MazG has a role in cellular 'house-cleaning' by removing abnormal (d)NTP's from nascent DNA strands (Galperin *et al.*, 2006), in addition to regulating programmed cell death in



*E. coli* (Gross *et al.*, 2006). It also regulates intracellular levels of (p)ppGpp, the main nutritional stress signal molecule involved in the stringent response, which has been shown to be an important response in *Campylobacter jejuni* during intestinal colonization (Stintzi *et al.*, 2005). By reducing (p)ppGpp levels, MazG has a central role in maintaining cell viability during nutritional stress, which could be important in the gut during periods of intermittent availability of certain nutrients, whereas (p)ppGpp itself is required for growth during osmotic stress in *L. monocytogenes* (Okada *et al.*, 2002). Moreover, recent work has identified a role for MazG in the mycobacterial oxidative stress response and virulence (Sasseti and Rubin, 2003; Lu *et al.*, 2010). As outlined above MazG has a number of different roles in different bacteria, but it is clear that stress response is a common theme. This study provides evidence for a novel role of MazG in salt tolerance. In the context of the gut environment, MazG may be important in removal of mutagenic nucleotides from growing DNA strands. Damage to bacterial DNA is likely due to exposure to genotoxic compounds, such as nitrosamines and heterocyclic amines (Kurokawa *et al.*, 2007). In addition, MazG could provide energy to cell during stress through the hydrolysis of ATP. Because of the numerous roles of MazG in different stress conditions, it may function as general stress response protein in the bacterial cell. More research and discovery of novel MazG proteins will help identify new physiological roles, substrates and precise mechanisms of action for these proteins in the myriad of stress conditions imposed on microorganisms.

In conclusion, we have identified five genetic loci involved in salt tolerance from within the human gut microbiome using a functional metagenomic approach. The genes represent two different homologues of *galE* and two of *mazG*, as well as a *murB* homologue, from three different species of the genus *Collinsella*, *Akkermansia* and *Eggerthella*. The identification of three genes within approximately 40 kb of metagenomic DNA from SMG 3 (highest genetic identity to *C. aerofaciens*) is relevant in that functionally related proteins are often co-located on the chromosome of prokaryotic genomes (Sleator and Walsh, 2010; Sleator, 2011). In addition to expanding our knowledge of salt tolerance mechanisms, this study may also facilitate the development of novel drug targets and related approaches to control resident gut microbiota (Sleator, 2010a, b). Ultimately some of the salt tolerance mechanisms identified might be used as part of a 'pathobiotechnology' (Sleator, and Hill, 2006) or 'metabiotechnology' (Culligan *et al.*, 2009) strategy for the design of improved probiotic cultures with greater resistance to process induced stresses (such as spray and freeze drying), as well as improved gut colonization (Sheehan *et al.*, 2006; Sheehan *et al.*, 2007; Watson *et al.*, 2008 for such examples). This will hopefully result in a broader and more

comprehensive representation of salt tolerance mechanisms in this unique environment.

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