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Evolutionary history and spread of multidrug resistant IncHI1 plasmids in *Salmonella* Typhi

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Abstract

Typhoid fever, infection with *Salmonella enterica* serovar Typhi (*S. Typhi*), remains a serious health concern in many parts of the world, particularly where sanitation is poor. While vaccines against *S. Typhi* are available, coverage in endemic regions is low, leaving antimicrobial therapy central to disease control. Unfortunately, drug resistant *S. Typhi* emerged in the mid-1970s and multi-drug resistance (MDR) is now a significant problem in many typhoid endemic areas. In the *S. Typhi* population, MDR is almost exclusively conferred by self-transmissible IncHI1 plasmids carrying a suite of antimicrobial resistance genes. We compared eight IncHI1 plasmid sequences [and](#) identified single nucleotide polymorphisms (SNPs) in the plasmid backbone. These were combined with known *S. Typhi* chromosomal SNPs to simultaneously genotype IncHI1 plasmids and their *S. Typhi* hosts among a collection of 454 *S. Typhi* dating back to 1958. Among isolates collected prior to 1995, a variety of IncHI1 plasmid types were observed, present in distinct *S. Typhi* hosts. Some plasmids were detected among distinct *S. Typhi* lineages co-circulating in time and space, indicating the spread of MDR via transfer of plasmids among *S. Typhi* strains. From 1995 onwards, 98% of MDR *S. Typhi* tested were of the same plasmid-strain combination (IncHI1 plasmid sequence type 6 (PST6) and *S. Typhi* haplotype H58) suggesting that the recent global spread of MDR typhoid is the result of clonal expansion of a single host-plasmid combination. In competition assays, the ability of PST6-bearing *S. Typhi* to outcompete *S. Typhi* bearing a distinct IncHI1 plasmid (PST1) varied depending on the host strain, suggesting plasmid-strain interactions may contribute to the recent success of the PST6 plasmid. Comparison of PST6 and PST1 in a common *S. Typhi* strain background revealed that PST6 conferred osmotolerance, which we demonstrate is due to the presence of the Tn6062 transposon incorporating *betU*.

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Introduction

Typhoid fever remains a serious public health problem in many developing countries, with highest incidence in Asia (274 per 100,000 person-years) and Africa (50 per 100,000 person-years) (1). Worldwide, the annual typhoid fever burden is estimated at more than 20 million cases and half a million deaths (2). The causative agent of typhoid fever is the bacterium *Salmonella enterica* serovar Typhi (*S. Typhi*). While vaccines against *S. Typhi* exist, it is mainly travellers (3, 4) and people enrolled in large vaccine trials (5) who are immunized, and antimicrobial treatment remains central to the control of typhoid fever (3). However antimicrobial resistant typhoid has been observed for over half a century and is common in many areas.

Chloramphenicol resistant *S. Typhi* was first reported in 1950, shortly after the drug was introduced for treatment of typhoid (6). By the early 1970s, *S. Typhi* resistant to both chloramphenicol and ampicillin had been observed (7), and multidrug resistant (MDR) *S. Typhi* (defined as resistant to chloramphenicol, ampicillin and trimethoprim -sulfamethoxazole) emerged soon after (8). In response, the recommended treatment for typhoid was changed in the mid 1990s to the fluoroquinolones (3, 9). However susceptibility to these drugs has been declining among *S. Typhi* ever since (10). The rate of MDR among *S. Typhi* isolated from patients fluctuates over time and geographical space, as does the precise combination of drug resistance genes and phenotypes (10, 11). However in many typhoid endemic areas, an increase in MDR *S. Typhi* was observed in the late 1990s (12-14). MDR typhoid remains a significant problem in many areas (10, 15), including Asia (e.g. >50% of cases in Cambodia in 2006-2009 (16) and Mekong Delta, Vietnam in 2004-2005 (17)), parts of Africa (e.g. 70% of cases in Nairobi, Kenya in 2004-2006 (18)) and the Middle East (e.g. 50% of cases in Jordan in 2004-2005 (19)). MDR is also

common among travellers returning to the UK, US or Canada with typhoid fever (20-22). MDR *S. Typhi* with reduced susceptibility to fluoroquinolones are now frequently observed (10, 16, 17, 23), leaving macrolide (azithromycin) or third generation cephalosporins as the only effective therapy (24, 25).

MDR among *S. Typhi* is almost exclusively conferred by self-transmissible plasmids of the HII incompatibility type (IncHI1) (8, 12, 26-31), although other plasmids are occasionally reported (32). In the laboratory, IncHI1 plasmids can transfer between *Enterobacteriaceae* and other Gram-negative bacteria (33) and in nature, IncHI1 plasmids have been detected in enteric pathogenic strains of *Salmonella* and *E. coli* (34-37).

The question of whether the spread of MDR pathogens is due to spread of resistance genes/plasmids among circulating pathogens, or to the expansion of MDR pathogen clones, is of public health interest. The latter implies that MDR cases are linked epidemiologically and the increase in MDR cases could potentially be halted by interrupting introduction or transmission of the MDR clone(s). The former suggests that MDR is acquired frequently, implying strong selective pressure for the MDR phenotype. For example, it was recently demonstrated that the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) ST5 around the world was due to frequent acquisition of the methicillin-resistance island by local ST5 strains, rather than to global spread of a single MRSA ST5 strain (38). This is informative in that it suggests that local patterns of antimicrobial use and infection control, rather than international transmission routes, should be the focus of interventions to reduce MRSA rates. In contrast, a study of *Neisseria gonorrhoeae* in Israel found that

quinolone resistant cases were the result of occasional imports of resistant strains into the local community rather than acquisition of resistance by endemic strains (39). The MRSA ST5 study used SNP typing to differentiate among the MRSA population at high resolution (38). In *S. Typhi*, efforts have been made to investigate variability among IncHI1 plasmids (30, 34, 40) or among *S. Typhi* host strains (23, 41-45), but little progress has been made in linking the two together to answer fundamental questions of how MDR typhoid spreads. Several lines of evidence indicate that MDR IncHI1 plasmids have transferred into wildtype *S. Typhi* populations on multiple occasions. Plasmid-associated antimicrobial resistance phenotypes and resistance gene content vary among *S. Typhi* isolates, as do RFLP profiles of IncHI1 plasmids found in *S. Typhi* (30). We recently developed a multi-locus sequence typing (MLST) scheme for IncHI1 plasmid sequences, which identified eight distinct IncHI1 plasmid sequence types (PSTs) among plasmids from *S. Typhi* and *S. Paratyphi A* isolates (40), including five PSTs found in *S. Typhi* (40). Phylogenetic analysis of the sequenced loci divided the PSTs into two distinct lineages, both of which were found in *S. Typhi* (40). This pattern is not consistent with a single acquisition of IncHI1 plasmid in *S. Typhi* followed by evolution into multiple plasmid lineages, rather it indicates that IncHI1 plasmids have entered the *S. Typhi* population on at least two independent occasions. However the phylogenetic relatedness of the host *S. Typhi* strains was not determined, thus we were unable to estimate how many acquisitions may have occurred.

In this study, we began with a comparative analysis of all available IncHI1 plasmid sequences (N=8) and inferred the timing of independent insertions of drug resistance-related transposons in IncHI1 plasmids. We then incorporated the SNPs identified in

this analysis into a high-throughput SNP typing assay, to simultaneously interrogate both IncHI1 plasmid and *S. Typhi* chromosomal sequences in a global collection of over 450 *S. Typhi* isolated during the last 50 years. Using this data, we investigated the historical acquisitions of IncHI1 in natural *S. Typhi* populations. Finally we investigated the role of the specific plasmid in this clonal expansion, using a combination of phenotype arrays, sequence comparison and gene constructs.

Results

Evolution of MDR IncHI1 Plasmids

We compared the sequences of eight ~200 kbp IncHI1 plasmids (Table 1). The plasmids shared a conserved IncHI1 backbone (>99% identical at the nucleotide level), including the *tra1* and *tra2* regions encoding conjugal transfer, as detected in previous analyses of 2-3 IncHI1 sequences (30, 34, 40, 46). We identified 347 single nucleotide polymorphisms (SNPs) within this conserved backbone, which were used to construct a phylogenetic tree (Figure 1). We used a Bayesian inference method (implemented in BEAST (47)), which also estimates the divergence dates of internal nodes of the tree based on the known isolation dates for the plasmid sequences (48) (Figure 1). The tree topology is in agreement with that inferred previously using the plasmid MLST approach (40). The three recent *S. Typhi* sequences (isolated 2003-2004) were very closely related and correspond to the previously defined plasmid sequence type (PST) 6 (40) (Figure 1, red). According to our divergence date estimates, the *most recent common ancestor* (mrca) shared by these three plasmids existed circa 1999 (Figure 1). The PST6 plasmids were also closely related to the PST7 plasmid pAKU_1 (Figure 1, orange), with mrca circa 1992. The plasmids pHCM1, pO111_1 and pMAK1 formed a distinct group corresponding to PST1, with mrca circa 1989 (Figure 1, green). The eighth plasmid, R27 (PST5), was quite distinct from the others, with an estimated divergence date of 1952 (Figure 1, black).

In addition to the conserved IncHI1 backbone, the plasmids each harbour insertions of drug resistance elements. These include transposons Tn10 (encoding tetracycline resistance), Tn9 (encoding chloramphenicol resistance via the *cat* gene), *strAB* (encoding streptomycin resistance), *sul1* and *sul2* (encoding sulfonamide resistance),

dfrA7 (encoding trimethoprim resistance) and *bla*_{TEM-1} (encoding ampicillin resistance), as described previously (30, 34, 49). The insertion sites of these elements, determined from sequence data and confirmed via PCR (Tables 2 & 3), differed between lineages of the IncHI1 tree (Figure 1). The insertion sites were conserved within lineages, enabling us to infer the branch on which each insertion occurred (Figure 1, grey). All plasmid sequences included Tn10, however three different insertion sites were evident (Table 2), indicating the transposon was acquired by IncHI1 plasmids on at least three separate occasions (Figure 1, grey). Tn9 was present in all plasmids ~~besides~~ except for R27, however the insertion site in PST6 and PST7 plasmids differed from that in PST1, indicating at least two independent acquisitions. It was previously noted that pHCM1 (PST1) and pAKU_1 (PST7) share identical insertions into Tn9 of a sequence incorporating Tn21 (including *sul1*, *dfrA7*), *bla*_{TEM-1}, *sul2*, and *strAB* (34); here we found this insertion into Tn9 was conserved in all PST1 and PST6 plasmid sequences. Together, this composite set of drug resistance elements encodes resistance to chloramphenicol, trimethoprim-sulfamethoxazole and ampicillin (the definition of MDR). These results provide strong evidence that MDR IncHI1 PST1 plasmids have spread the ~~MDR~~ phenotype among enteric pathogens including *S. Choleraesuis* (pMAK1), enterohaemorrhagic *E. coli* O111:H- (pO111_1) and some *S. Typhi* populations (pHCM1). Similarly, MDR IncHI1 PST6 and PST7 plasmids have spread the MDR phenotype among other *S. Typhi* populations and *S. Paratyphi A*.

Dissecting the Spread of MDR Typhoid

In order to investigate the role of IncHI1 plasmids in the historical and current spread of MDR *S. Typhi*, we performed high resolution genotyping of *S. Typhi* chromosomal

and IncHI1 plasmid loci in a global collection of 454 *S. Typhi*, isolated in 1958-2007 (Table 4, Table S1). These isolates include as controls 19 *S. Typhi* isolates sequenced previously (50). We also genotyped eight *E. coli* transconjugants harbouring IncHI1 plasmids of known plasmid sequence types previously described (30, 40) as additional PST controls. In order to increase our coverage of recent African isolates, the data presented here includes results from genotyping 22 *S. Typhi* isolated from Kenya in 2004-2007, which we reported previously (23). Genotyping was performed using two GoldenGate assays to simultaneously assay chromosomal and plasmid SNP loci. We targeted 230 loci in the conserved backbone of the IncHI1 plasmid (identified above, Table S2), 6 additional IncHI1 loci previously identified by plasmid MLST (40) and 119 loci associated with resistance genes and associated transposons (identified among the IncHI1 sequences analysed above) (17).

Of the 454 genotyped *S. Typhi*, 193 (43%) contained IncHI1 plasmids. These 193 IncHI1 plasmids clustered into nine distinct haplotypes, their phylogenetic relationships are shown in Figure 2b. The majority of IncHI1 plasmids gave positive signals for multiple resistance genes or elements including Tn10, Tn9, *strAB*, *sul1*, *sul2*, *dfrA7* and *bla*_{TEM-1}. Transposon insertion sites were confirmed for representative plasmids using PCR (Table 2) and conform with patterns of insertion sites determined from sequence data (Figures 1 & 2b). Only thirteen IncHI1 plasmids were identified in *S. Typhi* isolated prior to 1994 (Table 5), however these few plasmids include representatives of seven of the nine IncHI1 plasmid haplotypes.

The phylogenetic distribution of *S. Typhi* chromosomal haplotypes, which included 26 distinct clusters, is shown in Figure 2a. PST2 was found in three *S. Typhi*

haplotypes isolated in Asia between 1972 and 1977 (Table 5), consistent with repeated introduction of the same or very closely related IncHI1 plasmids into distinct *S. Typhi* lineages. PST8 was present in three *S. Typhi* isolated from Peru in 1981 (51). Two of these host strains were identical at all chromosomal loci assayed (H77) while the third host strain had a distinct haplotype (H50, which differed from H77 at 28 chromosomal SNP loci). It has previously been noted that these strains, and others collected contemporaneously at the same location, displayed distinct phage types (51). This is consistent with spread of the PST8 plasmid among multiple *S. Typhi* lineages co-circulating in Peru in the early 1980s. After 1994, nearly all IncHI1 plasmids were PST6 (180/184 plasmids, 98%). (Two PST2 plasmids and a novel subtype of PST1, 57Laos, were also detected after 1994; see Figure 2b, Table 3.) Remarkably, all of the 180 *S. Typhi* isolates carrying the PST6 plasmid were of the same chromosomal haplotype, H58. This strongly suggests that the apparent spread of MDR typhoid since the mid-1990s (12-14) is due to the clonal expansion of H58 *S. Typhi* carrying the MDR PST6 plasmid. This is in contrast to the historical data outlined above, in which closely related MDR plasmids appeared to spread among distinct co-circulating *S. Typhi* strains. The earliest *S. Typhi* H58 present in our collection was isolated in 1995 and carried the PST6 plasmid. Of the 390 *S. Typhi* isolated after 1994, 70% were of the H58 haplotype and 65% of these carried the PST6 plasmid (we found no *S. Typhi* H58 isolates carrying other IncHI1 plasmid types).

The clonal expansion of H58 *S. Typhi* has been documented previously (23, 44), however the role of the PST6 plasmid has not been investigated. To ascertain whether the common ancestor of *S. Typhi* H58 might have carried the PST6 plasmid, we

“zoomed in” to the phylogenetic structure among our 293 *S. Typhi* H58 isolates, described by 45 of the assayed SNP loci that differentiate within the H58 cluster (Figure 3). The isolates were divided into 24 distinct H58 subtypes, with the majority (N=270) in 13 haplotypes (Figure 3). Most of the H58 subtypes (N=14), including the ancestral subtype A, included isolates harbouring the PST6 plasmid (Figure 3, solid shading). We have previously sequenced the genomes of 19 *S. Typhi*, including seven isolates from the H58 haplogroup (50), and observed the insertion of an *IS1* transposase between protein coding sequences STY3618 and STY3619 within all sequenced H58 *S. Typhi* genomes. This transposase was identical at the nucleotide level to the *IS1* sequences within Tn9 in IncHI1 plasmids pHCM1 and pAKU_1, and shared a common insertion site in all seven H58 chromosomes sequenced (50). In the present study, our SNP assays included a probe targeting sequences within the *IS1* gene. Nearly all of the *S. Typhi* H58 isolates gave positive signals for this *IS1* target (Figure 3; coloured or white), with the sole exception of six isolates from the H58 ancestral node A, which also included three isolates that carried the PST6 plasmid and tested positive for *IS1* (Figure 3, node A). This suggests that the PST6 plasmid was likely acquired by the common ancestor of *S. Typhi* H58 (Figure 3, node A), followed by transposition of *IS1* into the *S. Typhi* chromosome, which is now conserved among all H58 strains tested. Thus the dominance of PST6 over other MDR IncHI1 plasmids (noted here and previously (40)) and the dominance of H58 over other *S. Typhi* haplotypes (noted here and previously (23, 44)) appears to be the result of a transcontinental clonal expansion of MDR, PST6-bearing, H58 *S. Typhi*.

Selective Advantages of IncHI1 PST6

These results suggest that the global spread of MDR typhoid since the mid-1990s is attributable to the spread of a single plasmid-strain combination. This raises the questions of why this particular combination has been so successful and why PST6 has remained so closely associated with *S. Typhi* H58, especially since we were able to transfer PST6 plasmid pSTY7 from *S. Typhi* to *E. coli* and back to *S. Typhi* (data not shown) and given the evidence for previous IncHI1 plasmids co-circulating and spreading between *S. Typhi* strains (above). Since the drug resistance phenotype conferred by PST6 ~~and~~ is indistinguishable from most other IncHI1 plasmids, we hypothesised that PST6 may provide its *S. Typhi* host with some additional selective advantage, over and above that of drug resistance. To investigate what this might be, we introduced representative PST1 (pHCM1) and PST6 (pSTY7) IncHI1 plasmids from Vietnamese *S. Typhi* isolates into a common host strain via conjugal transfer. To facilitate manipulation in the laboratory, we chose the laboratory strain BRD948 as our common host, which is an attenuated strain derived from the *S. Typhi* strain Ty2 (haplotype H10). BRD948 (PST1) was able to outcompete BRD948 (PST6), reaching a ratio of 3:1 after 4 days of competitive growth in LB broth (Figure 4a, black). We therefore hypothesized that the advantage conferred by PST6, if any, might be related to specific environmental conditions or to plasmid-strain interactions. To test the latter, we compared the growth of wildtype *S. Typhi* H1 (PST1) and *S. Typhi* H58 (PST6) isolated from typhoid patients in Vietnam and Pakistan (listed in Table 6). Both H58 (PST6) strains were able to outcompete the H1 (PST1) strain, so that *S. Typhi* H1 was barely detectable after four days of competitive growth (Figure 4a; red). However plasmid-free H58 *S. Typhi* were also able to outcompete plasmid-free H1 *S. Typhi* (Figure 4a; blue), thus we cannot confirm an H58-PST6 interaction.

To screen for conditions in which PST6 confers an advantage over PST1, we used Biolog phenotyping arrays to compare the growth of plasmid-free *S. Typhi* BRD948 to BRD948 (PST1) and BD948 (PST6) under a wide variety of conditions including various pH levels and osmotic/ionic strengths, and a wide variety of antibiotics and chemicals (52). As expected, both IncHI1 plasmids conferred enhanced growth in the presence of a wide range of antibiotics including amoxicillin, azlocillin, oxacillin, penicillin G, phenethicillin, chloramphenicol, streptomycin, gentamicin, tetracyclines, trimethoprim (Table S3). While neither plasmid conferred resistance to cephalosporins, the plasmid-bearing strains grew better than plasmid-free BRD948 in the presence of cephalosporins: cefazolin, cephalothin, and the third-generation cephalosporins cefotaxime, ceftriaxone and cefoperazone (Table S3), presumably due to the presence of the beta-lactamase gene *bla*_{TEM-1}. When tested for their minimum inhibitory concentration (MIC) to clinically relevant drugs all isolates were fully susceptible to cephalosporins (MICs \leq 0.125 to cefepime, cefotaxime and ceftazidime) and there was no evidence of extended spectrum beta-lactamase activity. Interestingly, BRD948 (PST1) was more resistant than BRD948 to a range of chemicals including the quinolones oxolinic acid and 5,7-Dichloro-8-hydroxy-quinaldine; fungicides acriflavine, benserazide, captan and tolylfluanid; and toxins sodium cyanate and zinc chloride, which may be due to non-specific efflux. Resistance to quinolones is a marker for reduced susceptibility to fluoroquinolones (53, 54), however the PST1 plasmid conferred no increase in MIC to the quinolone Nalidixic acid (MIC 2mg/L for BRD948, BRD948pHCM1 and BRD948pPST6); a similar lack of resistance (MIC 0.008 mg/L) was seen for ciprofloxacin. The only other difference between the two plasmid-bearing strains was that BRD948 (PST6) exhibited increased resistance to osmotic stress (3-5% NaCl or 6% KCl) (Table S3).

We hypothesised that the osmotolerant properties of PST6 may be explained by the presence of two putative transporters encoded within a composite transposon, Tn6062, found in PST6 but not PST1 plasmids (see Figure 5a; this transposon was called Ins1056 in (40)). Tn6062 is one of very few differences in gene content we identified between the sequenced plasmids pHCM1 (PST1) and pAKU_1 (PST7) (34, 40), and we have previously observed that Tn6062 was present in all IncHI1 plasmids of the PST6, PST7 and PST8 sequence types (40). In the present study, Tn6062 was detected by the positive signals of two loci within the SPAP0105 gene. All PST6 plasmids in our study carried Tn6062, as did the novel subtype of PST1 (57Laos) and two of the three PST8 plasmids (Peru, 1981) (Table 5). To determine if Tn6062 was responsible for the osmotolerance conferred on *S. Typhi* BRD948 by the PST6 plasmid, we cloned the two genes in Tn6062 (SPAP0105 and SPAP0196; see Figure 5a) into a low copy number vector pAYCY184 and assessed their effect on *S. Typhi* BRD948 in high salt concentration medium (0.8M NaCl LB broth, approx. 4.7% NaCl). *S. Typhi* BRD948 (PST1) was unable to grow under these conditions (Figure 5b; grey). However in the presence of PST6, the same host strain grew easily (Figure 5b; blue), confirming the phenotyping array result. BRD948 (pAYCY184-Tn6062) was able to grow at a slightly lower rate than BRD948 (PST6) (Figure 5b, blue), while BRD948 carrying the empty pAYCY184 vector was unable to grow at all (Figure 5b, black). Therefore the transposon Tn6062 carried by PST6 confers an osmotolerance phenotype on *S. Typhi* in addition to the MDR phenotype.

Discussion

The Spread of MDR Typhoid

Our analysis indicates that the IncHI1 plasmids responsible for the vast majority of MDR in *S. Typhi* are very closely related to IncHI1 plasmids causing MDR in other enteric pathogens. These plasmids share a recent common ancestor approximately six decades old and have evolved into several distinct lineages via a process of mutational divergence, followed by acquisition of resistance elements, then further mutational divergence (Figure 1). Simultaneous SNP typing of plasmid and host enabled us to differentiate between the clonal expansion of an MDR *S. Typhi* host strain, and independent acquisitions of the same MDR plasmids by distinct *S. Typhi* hosts. PST8 was found in *S. Typhi* of two distinct haplotypes, co-circulating in Peru in 1981, while PST2 was found in three distinct *S. Typhi* haplotypes in Asia between 1972-1976 (Table 5). This confirms that the spread of MDR typhoid in the 1970s and 1980s was due to the multiple acquisitions of IncHI1 plasmids in the *S. Typhi* population, involving multiple IncHI1 plasmids and the spread of individual plasmid types between *S. Typhi* haplotypes co-circulating in the same spatio-temporal location. One of the PST2-*S. Typhi* combinations (haplotype H42) was later detected among two strains from Africa in 2003-2004, suggesting that an individual IncHI1 plasmid may be able to persist in a single host strain for decades (Table 5). In stark contrast, all 193 PST6 plasmids were observed in *S. Typhi* of the H58 haplotype, and virtually all MDR *S. Typhi* observed after 1995 belonged to the same PST6-H58 clone, indicating that the recent global spread of MDR typhoid is the result of the clonal expansion of PST6-H58 *S. Typhi*. Since humans are the only known reservoir for *S. Typhi* (55), it is likely that trans-continental spread of this clone depends on international travel or

migration. If this is the case it will be particularly difficult to control since *S. Typhi* can be transmitted by asymptomatic carriers (56, 57), who are usually unaware of their status and are difficult to detect (58, 59).

The S. Typhi H58 - PST6 Association and Selection

Our data suggests that MDR among H58 *S. Typhi* is exclusively conferred by PST6 IncHI1 plasmids, and that this association can be explained by a single acquisition event in which the PST6 plasmid was acquired by the most recent common ancestor of *S. Typhi* H58 (Figure 3). Evidence from *IS1* and PST6 content of *S. Typhi* H58 isolates suggests that the common ancestor of H58 carried PST6, implying that the expansion of *S. Typhi* H58 did not begin until after acquisition of the plasmid (Figure 3). Evidence from sequence data indicates that the common ancestor of three PST6 plasmids sequenced from H58 strains in 2003-2004 existed around 1999 (Figure 1). This further supports the idea that PST6 was acquired once in H58 in the early 1990s, and its presence in current H58 isolates is due to co-segregation with its host strain as opposed to repeated introduction of PST6 plasmids into H58.

This tight association suggest PST6 may confer a strong selective advantage upon its H58 *S. Typhi* host. Since PST6 plasmids confer the same antimicrobial resistance phenotypes as other IncHI1 plasmids, it is unlikely that the expansion of H58 is due to selection for MDR alone. On the other hand, the PST6 MDR plasmid appears to have been lost from H58 *S. Typhi* in some areas where the switch to fluoroquinolones was made, including Nepal and Vietnam (42, 60, 61). Whereas, in locations where fluoroquinolones are not readily available and the use of chloramphenicol has been maintained for typhoid treatment, for example in Kenya, the plasmid has been

maintained in the *S. Typhi* population (18, 23). This confirms that antimicrobial use exerts a strong selective pressure for maintenance of the IncHI1 plasmid among *S. Typhi* and indicates that in the absence of such pressure, any additional advantages conferred (e.g. the increased osmotolerance we observed) is not enough to maintain the PST6 plasmid.

This highlights the dynamic and adaptive nature of antimicrobial resistance in bacterial pathogen populations, but it does not rule out the possibility that PST6 may have conferred an additional fitness advantage over other MDR IncHI1 plasmids when the selection for MDR was strong. Data from southern Vietnam in the mid-1990s suggests there was a shift from one IncHI1 plasmid (pHCM1; PST1), which had been circulating at high frequency in the local *S. Typhi* population, to another (pSTY7; PST6) in 1995 (30). The plasmid pHCM1 came from an *S. Typhi* H1 strain but we were unable to confirm the haplotype of the host strain from which pSTY7, the prototype PST6 plasmid, was isolated since the plasmid had been transferred into *E. coli* for storage. However since all 193 PST6 we detected were found in *S. Typhi* H58, including 135 isolates from Vietnam, it is highly likely that the original host of pSTY7 was H58. Thus the competition assays we performed, using PST6 plasmid-bearing H58 strains and PST1 plasmid-bearing H1 strains from Vietnam, may be a reasonable approximation for the competition that occurred in the endemic *S. Typhi* population in Vietnam in the early 1990s. The assays showed that, under laboratory conditions, both PST6-H58 *S. Typhi* tested were able to strongly outcompete a PST1-H1 strain (Figure 4a). However the role of the plasmid remains unresolved, since plasmid-free H58 *S. Typhi* were also able to outcompete plasmid-free H1 *S. Typhi* (Figure 4a).

Plasmid-Strain Interactions

To investigate strain-plasmid interactions thoroughly, extensive competition experiments involving numerous plasmid-bearing isolates (as independent biological replicates) and conjugal transfer of plasmids between strains of different haplotypes followed by extensive subculturing (to account for plasmid-strain adaptations), will need to be performed under a variety of growth conditions. This is beyond the scope of our study, however it is interesting to note that in the common strain background of BRD948 and under laboratory conditions, the situation was reversed such that PST1 appeared to have an advantage over PST6 (Fig. 5A; black). This was unexpected given the competitive advantage exhibited by wildtype PST6-H58 over PST1-H1 and the current real-world dominance of PST6. This could be due to plasmid-strain interactions, or the vast differences between laboratory growth conditions and the real-world niches in which *S. Typhi* needs to grow.

We initially used a common strain background to ensure that any observed differences were due entirely to the differences between PST6 and PST1 plasmids. However this may not be an accurate reflection of real-world plasmid competition dynamics. While the fitness of a particular host strain is usually reduced when a large plasmid such as IncHI1 is acquired, fitness can then increase as the plasmid and strain adapt to the new combination during subsequent generations, resulting in a fitness level equal to or greater than the original plasmid-free strain (62). This increased fitness can be due to adaptations in the plasmid and/or the host strain during co-evolution over hundreds of generations (62, 63). While the PST1 and PST6 plasmids used in the experiments had been initially isolated in *S. Typhi*, they had been stored in

E. coli for some time prior to conjugal transfer into *S. Typhi* BRD948. It is therefore possible that the plasmids were no longer well-adapted to *S. Typhi*, or that BRD948 was not well adapted to the IncHI1 plasmids. After introduction of the plasmids to BRD948, the recipient strains were kept in glycerol stocks prior to performing competition assays, thus our experiments may not accurately reflect the real-world situation in which new plasmid-host combinations are able to equilibrate over many generations, resulting in fitter strains (62). It is also possible that *S. Typhi* BRD948, which is attenuated by a deletion of *aroC*, *aroD* and *htrA* and was derived from a strain that has been grown in the laboratory for many decades (64), is not representative of *S. Typhi* currently circulating in typhoid endemic areas.

Tn6062 and Osmotolerance

Despite the uncertainties surrounding strain-strain differences, plasmid-strain interactions and real-world selection pressures, our analyses revealed that PST6 also confers osmotolerance on its *S. Typhi* host, via Tn6062. The transposon Tn6062 is made up of two genes, *betU* and SPAP0105, flanked by *IS1* elements in direct orientation (Fig. 4A). The conserved gene SPAP0105 contains a signal peptide sequence and four probable transmembrane helices, suggesting it may be an outer membrane protein, however it contains no protein domains of known function. BetU contains a betaine-choline-carnitine transporter family domain and encodes a betaine uptake system, capable of transporting glycine betaine and proline betaine (65). It was first described in *E. coli* strains causing polynephritis (ascending urinary tract infection) and is believed to be an osmoregulator, allowing *E. coli* to survive the high osmolarity and urea content in urine (65). However the gene is distributed among *E.*

coli with a range of pathogenic phenotypes, so its osmoprotectant properties may be useful in other environmental contexts (66).

It is possible that enhanced osmotolerance may ~~enhance~~ facilitate the survival of *S. Typhi* in specific niches ~~inside~~ within the human body, including the gut, gall bladder, urinary tract or liver, or in the external environment. *S. Typhi* invades via the intestinal epithelium (67) and can establish chronic asymptomatic carriage in the gall bladder (68). The bacterium is shed in faeces (68) and urine (69) and has recently been detected in the liver of chronic carriers (70). *S. Typhi* can be transmitted via contaminated water, but it can be difficult to culture from water samples. Little is known about the mechanisms required for survival of *S. Typhi* in the environment. It is possible that the ability to grow in the presence of high salt concentrations might enhance the ability of *S. Typhi* to continue replicating in certain environments outside the host, which may lower the infectious dose or enhance the possibility of transmission by increasing the level of *S. Typhi* contamination in a given environment.

Conclusions

The global spread of MDR typhoid since the mid-1990s is most likely due to clonal expansion of a single plasmid-strain combination, namely *S. Typhi* H58 bearing the IncHI1 PST6. This stands in stark contrast with patterns of MDR typhoid in the 1970s-1980s, when distinct IncHI1 plasmids were present in distinct *S. Typhi* hosts and spread between co-circulating *S. Typhi* lineages. The success of the H58-PST6 clone remains unexplained, however we found PST6 plasmids conferred the additional phenotype of osmotolerance via Tn6062, which may contribute to the selection of PST6 over other IncHI1 plasmids previously circulating among *S. Typhi*.

Materials and Methods

Bacterial isolates and DNA extraction

The bacterial isolates analysed by SNP array are summarized in Table 1 and listed in full in Supplementary Table 1. DNA was extracted using Wizard Genomic DNA purification kits (Promega) according to manufacturer's instructions. Details of the four isolates used for competition experiments are listed in Table 5.

BRD948 is the attenuated Ty2-derived strain CVD908-*htrA*, which has deletion mutations in *aroC*, *aroD*, and *htrA* (64). The growth of BRD948 on LB agar or in LB broth was enabled by supplementation with aromatic amino acid mix (aro mix) to achieve the final concentration of 50 μM L-phenylalanine, 50 μM L-tryptophan, 1 μM para-aminobenzoic acid and 1 μM 2,3-dihydroxybenzoic acid.

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Identification and phylogenetic analysis of IncHI1 SNPs

Plasmid sequences listed in Table 1 were downloaded from EMBL. SNPs between finished plasmid sequences were identified using the *nucmer* and *show-snps* algorithms within the MUMmer 3.1 package (71), via pairwise comparisons against pAKU_1 as the reference sequence. To identify SNPs in *S. Typhi* PST6 IncHI1 plasmids, 36 bp single-ended Illumina/Solexa sequencing reads from *S. Typhi* strains E03-9804, ISP-03-07467 and ISP-04-06979 were aligned to the pAKU_1 sequence using Maq (72) and quality filters as described previously in (50). SNPs called in repetitive regions or inserted sequences were excluded from phylogenetic analysis, so that phylogenetic trees were based only on the conserved IncHI1 backbone sequence. This resulted in a total of 347 SNPs, which were analysed using BEAST (47) to simultaneously infer a phylogenetic tree and divergence dates (using the year of isolation of each plasmid as listed in Table 1, resulting tree in Figure 1). Parameters used were as follows: generalised time reversible model with a Gamma model of site heterogeneity (4 gamma categories); a relaxed molecular clock with uncorrelated exponential rates (47), a coalescent tree prior estimated using a Bayesian skyline model with 10 groups (73), default priors, 20 million iterations.

SNP typing analysis

The chromosomal haplotype of *S. Typhi* isolates was determined based on the SNPs present at 1,485 chromosomal loci identified previously from genome-wide surveys (44, 50) and listed in (23, 42). IncHI1 plasmid haplotypes were determined using 231 SNPs located in the conserved IncHI1 backbone sequence, listed in Table S2 (note these do not include SNPs specific to pMAK1 or pO111_1 which were not available at the time of assay design; however additional SNPs identified via plasmid MLST

(40) were included, see Table S2). Resistance gene sequences were interrogated using additional oligonucleotide probes, listed in (45). All loci were interrogated using a GoldenGate custom array according to the manufacturer's standard protocols (Illumina), as described previously (23, 42, 45). SNP alleles were concatenated to generate two multiple alignments, one each for chromosomal and IncHI1 plasmid SNPs. Maximum likelihood phylogenetic trees (Figures 2-3) were fit to each alignment using RAxML (74) with a GTR+ Γ model and 1,000 bootstraps.

PCR

PCR primers were designed using Primer3 (75) according to the following criteria: melting temperature 56°C, no hairpins or dimers affecting 3' ends, no cross-dimers between forward and reverse primers. Primer sequences are given in Table 3. PCRs were performed on a TETRA DNA Engine Peltier Thermal Cycler (MJ Research) with a reaction consisting of 1.2 μ l ~~10X~~ Mango PCR buffer ~~10x~~, 1.5 mM ~~of~~ MgCl₂, 25 μ M of each dNTP, 1.25 U ~~of~~ Mango *Taq* (Bioline), 0.3 μ M of each primer, 1.0 μ l ~~of~~ DNA template (approx. 100 ng) and nuclease free water ~~to-in a~~ the total reaction volume of 12 μ l. Cycling conditions were as follows: 5 min at 94°C, 30 cycles of 15s at 94°C, 15s at 58°C, and 60s at 72°C; final extension of 5 min at 72°C.

Plasmid transfer

The transfer of pHCM1 and pSTY7 from respective *E. coli* transconjugants to the attenuated *S. Typhi* BRD948 was performed by cross-streaking onto LB agar supplemented with aro mix and incubating at 37°C overnight. The growth was harvested, resuspended in 2 ml of dH₂O, plated on MacConkey agar containing streptomycin (1 μ g/ml or 5 μ g/ml) and chloramphenicol (5 μ g/ml or 20 μ g/ml) and

incubated overnight at 37°C. BRD948 transconjugants were confirmed by antimicrobial susceptibility patterns (disk diffusion) and colony PCR specific for BRD948 background (primers 5939-5'-CGTTCACCTGGCTGGAGTTTG-3' and 5940-5'-CATGCCAGCAGCGCAATCGCG-3') and pHCM1 or pSTY7 plasmids (Insert1056L- 5'-TAGGGTTTGTGCGGCTTC-3' and Insert1056R-5'-CCTTCTTGTCGCCTTTGC-3').

Competition assays in common strain background

The competition between BRD948 (pHCM1) and BRD948 (pSTY7) was started in equal inoculums of roughly 5×10^3 CFU each in 10 mL of LB broth supplemented with aro mix and chloramphenicol (5 µg/mL). The culture was incubated for 16 hours at 37°C with shaking. Approximately 10^4 CFU of this culture were then used to inoculate the next passage. The cultures were passaged for a total of 4 days. Samples were collected at time point 0 (at the time of initial inoculation) and after 1, 2, 3 and 4 days of passage, diluted and spread on LB agar supplemented with aro mix. Sixty-four colonies from each sample were randomly picked and tested by PCR to identify their plasmid type (see below). The entire competition assay was performed in triplicate, i.e. beginning with three initial cultures of equal inoculums of the two strains. The colony PCR was performed using standard condition (see PCR section above) with three primers HCM1.DF 5'-CGATTTGTGAAGTTGGGTCA-3', HCM.DR2 5'-CAACCTGGGCAGGTGTAAGT-3' and HCM.DR3 5'-TTCGTTACGTGTTTCATTCCA-3'. Expected sizes of PCR products were 511 bp for BRD948 (pHCM1) and 285 bp for BRD948 (pSTY7).

Competition assays using wildtype strains

Eight individual competitive growth assays were performed; H58c vs. H58e, H58c vs. H1, H58e vs. H1, H58c-ST6 vs. H58e-ST6, H58c-ST6 vs. H1-ST1, H58e-ST6 vs. H1-ST1, H58c-ST6 vs. H58e and H58e-ST6 vs. H58c. The corresponding strains for each of the haplotypes and plasmid types is shown in Table 5. Bacterial isolates were recovered from frozen stocks onto Luria-Bertani (LB) media, supplemented with 20 mg/ml of chloramphenicol for strains with MDR plasmids. Individual colonies were picked and used to inoculate 10 ml of LB broth, which were incubated overnight at 37°C with agitation. Bacterial cells were enumerated the following day by serial dilution and plating. Equivalent quantities of the two competing *S. Typhi* strains were inoculated into 10 ml of LB broth and were incubated as before (Day 0). The competition assays were conducted by growing the mixed bacteria to stationary phase and then passaging them into 10 ml of LB broth in a 1:1000 dilution in triplicate over six days. The total colony forming units per ml were calculated for each assay by serial dilution and plating on LB media with and without chloramphenicol (20 mg/ml) to determine the proportion of strains with and without plasmids on Day 6. Additionally, 1 ml of media containing bacteria from each of the triplicates was stored at -80°C at each time point. DNA was extracted from the frozen samples by boiling for 10 minutes, samples were centrifuged at 8,000 rpm [give centrifugal speed in x g] in a microfuge for 5 minutes. The supernatant was removed and used as template in all of the subsequent competitive real-time PCR reactions, which were performed on each template in duplicate.

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Real-time PCR for quantitation of wildtype strains in competition assays

We performed two individual competitive real-time PCRs (Taqman system) with LNA probes to calculate the proportions of *S. Typhi* H1 vs. *S. Typhi* H58 and *S.*

Typhi H58c vs. *S. Typhi* H58e. These assays were performed to accurately calculate the relative proportion of the strains in all competitive assays, including those that could not be calculated by plating alone. The haplotype specific primers and probes were designed using Primer Express Software (Applied Biosystems) and manufactured by Sigma-Prologo (Singapore). Primer and probe sequences were as follows; (capital letters indicate the position of LNA and the bold letters indicate the SNP position) H58 vs H1 (99 bp amplicon): F(71-83)-CCGAACGCGACGG, R(169-157)-TGCGGCACACGGC and probe 5'-FAM-ccggtAat**G**gtAatGaagc-BHQ1 (*S. Typhi* H1) and 5'-Hex-ccggtAat**A**gtAatGaagc (*S. Typhi* H58); H58c vs H58e (89 bp amplicon): F(60-75)-ACCCTGCACCGTGACC, R-(148-135)-GCATGATGCCGCC and probe 5'-FAM-ttcCag**G**ccAtgAcgc –BHQ1 (*S. Typhi* H58c) and 5'-HEX-ttcCag**A**ccAtgAcgc-BHQ1 (*S. Typhi* H58e). PCR amplification were performed using a light cycler (Roche, USA), with hot start Taq polymerase (Qiagen, USA) under the following conditions, 95°C for 15 minutes and 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. As the primer locations were identical for the internal competitive PCR assay, the efficiency of the PCR was also considered to be identical. Therefore, proportions of strains at the various time points throughout the assay were calculated by taking the mean of six *C_p* values (each competition assay was performed in triplicate and the PCR was performed in duplicate). The Mean *C_p* values for each competitive assay was converted into a proportion (strain A) using the following calculation: Proportion strain A = $1/(2^{-\Delta C_p} + 1)$, where $\Delta C_p = C_p(\text{strain B}) - C_p(\text{strain A})$.

Phenotype Microarrays

Phenotype microarrays of osmotic/ionic response (PM 9), pH response (PM 10) and bacterial chemical sensitivity (PM 11 to 20) were performed as described previously by Biolog Inc. (Hayward, California USA) (52). BRD948 was used as a reference for comparison with BRD948 (pHCM1) or BRD948 (pSTY7) test strains to identify the phenotypes affected (either gained or loss) by the presence of IncHI1 plasmid pHCM1 (ST1) or pSTY7 (ST6).

The three strains were pre-grown on LB (Luria-Bertani) agar plates supplemented with 1X of an aromatic amino acid mix (a 50X aromatic amino acid mix consisted of 50 μ M L-phenylalanine, 50 μ M L-tryptophan, 1 μ M para-aminobenzoic acid and 1 μ M 2,3-dihydroxybenzoic acid). Sterile cotton swabs were used to pick colonies and suspend them in 10 ml IF-0a (Biolog), the optical density of which was then adjusted to 0.035 absorbance units at 610 nm. A total of 750 μ l of this cell suspension was diluted 200 fold into 150 ml IF-10 (Biolog), containing 1X aromatic acid mix. PM microtitre plates 9-20 were inoculated with 100 μ l of the cell suspension and 100 μ l of Biolog media supplemented with aromatic amino acid (containing 1.2X of Biolog media, 22 ml of sterile water and 3 ml of 50X aromatic amino acid mix) per well. Microtitre plates were then incubated at 37°C for 48 h in the Omnilog (Biolog Inc) and each well was monitored for colour change (kinetic respiration). Tests were performed in duplicate and the kinetic data was analysed using the OmniLog PM software set (Biolog Inc). A lower threshold of 80 omnilog units (measured as area under the kinetic response curve) was set, and the phenotypes of each of the three strains were compared.

Cloning and growth curves

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The fragment of two CDSs within Tn6062 of pSTY7 (3405 bp) was amplified using two primers IS1056-03 (5'-CAGGCACCGTTTTCTTATTAGAATCTTCGCCACT-3') and IS1056-04 (5'-TCATTGAACTTTGCTACCCTGA-3'). The pACYC184 fragment (2033 bp) containing its p15A *ori* and chloramphenicol resistant gene (*cmR*) was amplified using pACYC184-01 (5'-AAAATTACGCCCCGCCCTGC-3') and pACYC184-03 (5'-TAATAAGAAAACGGTGCCTGACTGCGTTAGCA-3'). The two fragments were then fused together by overlapping primer extension PCR (pACYC184-03 and IS1056-03 were two overlapping primers) using pACYC-01 and IS1056-04 primers. All three PCRs above were performed using *PfuUltra* II Fusion HS DNA Polymerase (Agilent, former Stratagene, UK) to achieved highly accurate amplification. The PCRs were set up following the manufacturer's manual with the specific annealing temperature of 58°C and extension time of 45s for Tn6062 and pACYC184 fragments or 1.5 min for the fusion fragment. The fused PCR product was re-circularised by T4 ligase (New England BioLabs, UK) to form pACYC184Δtet::Tn6062 and electroporated to BRD948. The pACYC184 fragment was also re-circularised to form the empty vector pACYC184Δtet and electroporated to BRD948.

Overnight bacterial cultures of BRD948 (pHCM1), BRD948 (pSTY7), BRD948 (pACYCΔtet) and BRD948 (pACYCΔtet::Tn6062) were diluted by distilled water to the cell suspension of 0.1 OD₆₀₀ before 1 μl of the cell suspension was inoculated into 200 μl of 0.8M NaCl LB broth (supplemented with aromatic amino acid mix) in a well of a 96-well plate. Each strain was inoculated to 6 wells (6 biological replicates). The bacteria were grown at 37°C with shaking at 300 rpm and OD₆₀₀ was measured automatically every 15 minutes for 24 hours in the Optima plate reader (BMG

Labtech, Germany). The absorbance data were collected and saved in Excel format for graphing.

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Plasmid	Host	Year of isolation	Plasmid type	Accession	Citation
pHCM1	<i>S. Typhi</i> strain CT18	1993	PST1	AL513383	(49)
pAKU_1	<i>S. Paratyphi A</i> strain AKU_12601	2003	PST7	AM412236	(34)
R27	<i>S. Typhimurium</i>	1961	PST5	AF250878	(76)
pMAK1	<i>S. Choleraesuis</i> strain L-2454	2002	PST1	AB366440	-
pO111_1	<i>E. coli</i> O111:H- strain 11128	2001	PST1	AP010961	(77)
p9804_1	<i>S. Typhi</i> strain E03-9804	2004	PST6	ERA000001	(50)
p7467_1	<i>S. Typhi</i> strain ISP-03-07467	2003	PST6	ERA000001	(50)
p6979_1	<i>S. Typhi</i> strain ISP-04-06979	2004	PST6	ERA000001	(50)

Table 1. IncHI1 plasmid sequences analysed in this study.

IncHI1 plasmid sequence type	PST1		PST5	PST6	PST6	PST7	PST8		
Plasmid or isolate	pHCM1	pMAK1	p0111_1	R27	p6979	pSTY7	pAKU1	81918	8186
Bacterial host	T	C	Ec	Tm	T	T	Pa	T	T
<i>Tn10</i> insertion	<i>B</i>	<i>B</i>	<i>B</i>	<i>C</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>
sequence data	<i>B</i>	<i>B</i>	<i>B</i>	<i>C</i>	<i>A</i>	<i>n/a</i>	<i>A</i>	<i>n/a</i>	<i>n/a</i>
N (<i>Tn10</i> - HCM1.247)	yes	n/d	n/d	no	no	no	no	no	no
O (<i>tetD</i> - SPAP0276)	no	n/d	n/d	no	yes	yes	yes	yes	yes
P (SPAP0261 - <i>Tn10</i>)	no	n/d	n/d	no	yes	yes	yes	yes	yes
<i>Tn9</i> insertion	<i>B</i>	<i>B</i>	<i>B</i>	<i>no</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>no</i>	<i>A</i>
sequence data	<i>B</i>	<i>B</i>	<i>B</i>	<i>no</i>	<i>A</i>	<i>n/d</i>	<i>A</i>	<i>n/a</i>	<i>n/a</i>
J (<i>cat</i> - <i>trhN</i>)	no	n/d	n/d	no	yes	yes	yes	no	yes
K (<i>mer</i> - <i>trhI</i>)	no	n/d	n/d	no	yes	yes	yes	no	yes
M (<i>cat</i> - HCM1.203)	yes	n/d	n/d	no	no	no	no	no	no
L (<i>insA</i> - <i>tetA</i>)	yes	n/d	n/d	no	no	no	no	no	no
<i>Tn21</i> into <i>Tn9</i>	<i>yes</i>	<i>yes</i>	<i>yes</i>	<i>no</i>	<i>yes</i>	<i>yes</i>	<i>yes</i>	<i>no</i>	<i>yes</i>
sequence data	<i>yes</i>	<i>yes</i>	<i>yes</i>	<i>no</i>	<i>yes</i>	<i>n/d</i>	<i>yes</i>	<i>n/a</i>	<i>n/a</i>
H (<i>tnpA</i> - <i>Tn9</i>)	yes*	n/d	n/d	no	yes	yes	yes	no	yes
I (<i>merR</i> - <i>Tn9</i>)	yes*	n/d	n/d	no	yes	yes	yes	no	yes
bla/sul/str into <i>Tn21</i>	<i>yes</i>	<i>yes</i>	<i>yes</i>	<i>no</i>	<i>yes</i>	<i>yes</i>	<i>yes</i>	<i>no</i>	<i>yes</i>
sequence data	<i>yes</i>	<i>yes</i>	<i>yes</i>	<i>no</i>	<i>yes</i>	<i>n/d</i>	<i>yes</i>	<i>n/a</i>	<i>n/a</i>
G (<i>strB</i> - <i>tniDelta</i>)	yes	yes	yes	no	yes	n/d	yes	no	yes
<i>strAB</i> 2 nd copy	<i>no</i>	<i>no</i>	<i>no</i>	<i>no</i>	<i>no</i>	<i>no</i>	<i>yes</i>	<i>yes</i>	<i>yes</i>
sequence data	<i>no</i>	<i>no</i>	<i>no</i>	<i>no</i>	<i>no</i>	<i>n/d</i>	<i>yes</i>	<i>n/a</i>	<i>n/a</i>
Q (<i>strB</i> - SPAP0228)	no	n/d	n/d	no	no	no	yes	yes	yes

[\[using + and – designations in the table might be easier?\]](#)

Table 3. Resistance gene insertion sites in IncHI1 plasmids inferred from a combination of PCR and sequencing. Summaries of five insertion patterns are shown in bold italics; these are inferred from sequence data where available (italics) and PCR using primers shown in Table 3 (labelled G-Q). T = *Salmonella enterica* serovar Typhi, C = *Salmonella enterica* serovar Choleraesuis, Tm = *Salmonella enterica* serovar Typhimurium, Pa = *Salmonella enterica* serovar Paratyphi A, Ec = *E. coli* O111:H-. *Distinct amplicon size for PST1; n/d PCR not done; n/a sequence

data not available. “*strAB* 2nd” copy refers to the insertion of streptomycin resistance genes *strAB* directly into the plasmid backbone, not as part of the *bla/sul/str* element.

	Forward primer Reverse primer	Length in pAKU_1 (bp)	Length in pHCM1 (bp)
G	GATGGAGAAGAGGAGCAACG TTCGTTCCCTGGTTCGATTTTC	989	989
H	GTGCTGTGGAACACGGTCTA TCATCAACGCTTCCTGAATG	271	1,598
I	ACGAAAGGGGAATGTTTCCT CGAGTGGGAATCCATGGTAG	163	1,490
J	CAAAATGTTCTTTACGATGCC CCAGACAGGAAAACGCTCA	2,219	none
K	CTGTGCCGAGCTAATCAACA ACGAAAGGGGAATGTTTCCT	1,314	none
L	TTTTAAATGGCGGAAAATCG GCCAGTCTTGCCAACGTTAT	none	1,872
M	GGGCGAAGAAGTTGTCCATA ATTCGAGCAAAACCATGGAA	none	2,195
N	CGGGATGAAAAATGATGCTT GGTCGGTGCCTTTATTGTTG	none	2,180
O	GCGTACAAAAGGCAGGTTTG GCTTGATGATGTGGCGAATA	1,823	none
P	TGGTCGGTGCCTTTATTGTT GGGCGTCAGAGACTTTGTTC	1,899	none
Q	TTCGCCCCGATATAGTGAAGG CTAACGCCGAAGAGAACTGG	1,923	none

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Table 3. PCR primers for detection of resistance gene insertion sites

Region	No. countries	pre-1970s	1970s-1980s	1990s	2000-2007	Total isolates
South & Central America	4	0	6	3	2	11
Central, Southern, East Africa	7	10	3	3	26	42
North Africa	3	11	1	8	5	25
West Africa	11	28	0	6	12	46
East Asia	8	5	8	22	187	222
Indian Subcontinent	3	0	3	1	66	70
Middle East	3	0	0	0	31	31
Europe	5	1	1	2	2	6
Unknown	-	1	0	0	0	1
<i>Total</i>	<i>44</i>	<i>56</i>	<i>22</i>	<i>45</i>	<i>331</i>	<i>454</i>

Table 4. Summary of 454 *S. Typhi* isolates analysed in this study.

Note full details of these *S. Typhi* isolates, and eight *E. coli* transconjugants, are given in Supplementary Table 1.

Isolate	Year	Country	Chr	Plas	ISI	cat	tetA	tetC	tetD	tetR	Tn10LR	impA	merAPRT	InlI	sulI	dhfr	dhfrA7	bla	IS26	sul2	strAB	betU
76-54	1976	Chile	H50	7654																		
78-851	1978	Tunisia	H9	78851																		
CT18	1993	Vietnam	H1	PST1																		
76-1406	1976	Indonesia	H42	PST2																		
75-2507	1975	India	H55	PST2																		
77-302	1977	India	H55	PST2																		
77-303	1977	India	H55	PST2																		
72-1907	1972	Vietnam	H68	PST2																		
72-1258	1972	Mexico	H11	PST3																		
73-1102	1973	Vietnam	H87	PST4																		
81-863	1981	Peru	H50	PST8																		
81-424	1981	Peru	H77	PST8																		
81-918	1981	Peru	H77	PST8																		
57Laos	2000*	Laos	H1	57Laos																		
03-4747	2003*	Togo	H42	PST2																		
04-6845	2004*	Benin	H42	PST2																		

Table 5. Chromosome, plasmid and resistance gene details of drug resistant *S.*

Typhi isolated up to 1993*. Colour indicates the presence of transposase or resistance-associated genes (given in columns) in each *S.* Typhi isolate (rows).

*Indicates the only MDR *S.* Typhi isolated after 1993 that were not of the H58 haplotype or PST6 IncHI1 haplotype.

Strain ID	Haplotype node	Plasmid	Year	Location
1727	H58-C	none	2004	Pakistan
BJ4	H58-E1	none	2004	Southern Vietnam
255	H58-C	PST6		Unknown?
AG45	H58-E1	PST6	2004	Southern Vietnam
TY36	H1	none	??	Southern Vietnam
DTC52	H1	PST1		Southern Vietnam

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Table 6. *S.* Typhi isolates used in competition assays. Haplotype nodes are labelled as in Figure 2a.

Figure legends

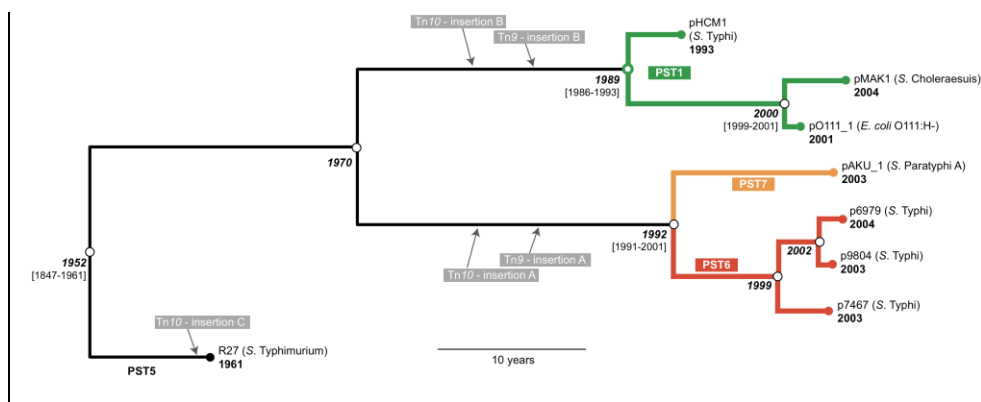


Figure 1. Phylogenetic tree for IncHI1 plasmid sequences.

Phylogenetic tree based on 347 SNPs identified among 8 publicly available IncHI1 plasmid sequences, constructed using BEAST (with 20 million iterations, 4 replicate runs, exponential clock model). Terminal nodes are labelled with the date of isolation, which were input into the BEAST model in order to estimate divergence dates for internal nodes (open circles, labelled with divergence date estimates; brackets indicate 95% highest posterior density interval). Insertion sites (grey) are based on sequence data and verified (except for pO111_1 and pMAK1) by PCR. Precise insertion sites and PCR primers for verification are given in Tables 2-3. Four major plasmid groups, PST1, PST5, PST6, PST7, are coloured as labelled.

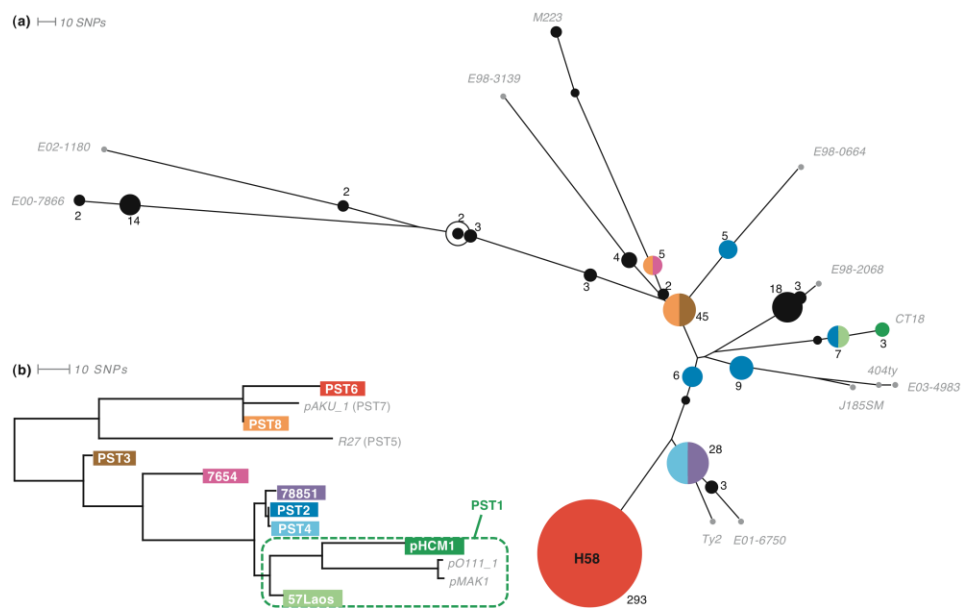


Figure 2. Phylogenetic trees of *S. Typhi* chromosome and IncHI1 plasmid.

(a) Phylogenetic tree indicating chromosomal haplotypes of 454 *S. Typhi* isolates.

Nodes indicate the detection of *S. Typhi* isolates, node sizes are scaled to the number of isolates detected, which is indicated with numeric labels. Unfilled circle indicates tree root; reference isolates used to define the *S. Typhi* SNPs are shown as grey nodes, labelled with the isolate name. Colours indicate the presence of IncHI1 plasmids within *S. Typhi* isolates of that haplotype; numbers represent the number of isolates at each node, different colours indicate different IncHI1 plasmid haplotypes corresponding to the tree in (b); black indicates no IncHI1 plasmid found in any strain of that particular chromosomal haplotype. Note that most of the nodes colour-coded to represent plasmid type also contain *S. Typhi* isolates with no plasmid, and the colours do not represent the proportion of strains harbouring the plasmid.

(b) Phylogenetic tree of IncHI1 plasmids for 201 genotyped MDR *S. Typhi*. Coloured leaf nodes represent plasmid haplotypes that have been found in *S. Typhi*.

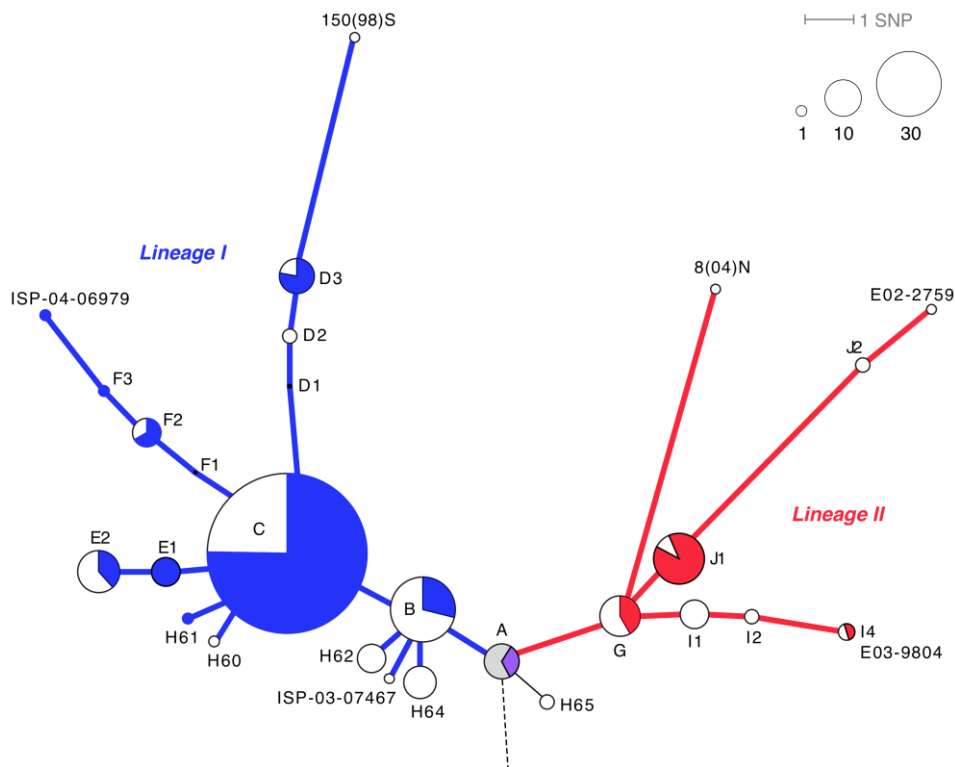


Figure 3. Phylogenetic tree of the H58 haplogroup of *S. Typhi*. Dashed line indicates where this tree joins into the larger phylogenetic tree of *S. Typhi* (shown in Figure 1). The two major H58 lineages are indicated by colour (blue, lineage I; red, lineage II; purple, common ancestor of both). Nodes are labelled with strain names (outer nodes representing sequenced strains; see (50)), haplotype (H followed by number) as defined in (44) or letters indicating nodes resolved by SNP typing. Node sizes indicate the relative frequency of each haplotype node within the study collection of 269 H58 *S. Typhi* isolates, according to the scale provided. The proportion of isolates in each node carrying the PST6 plasmid and IS1 (solid colour), IS1 only (white) or neither (grey) is indicated by shading.

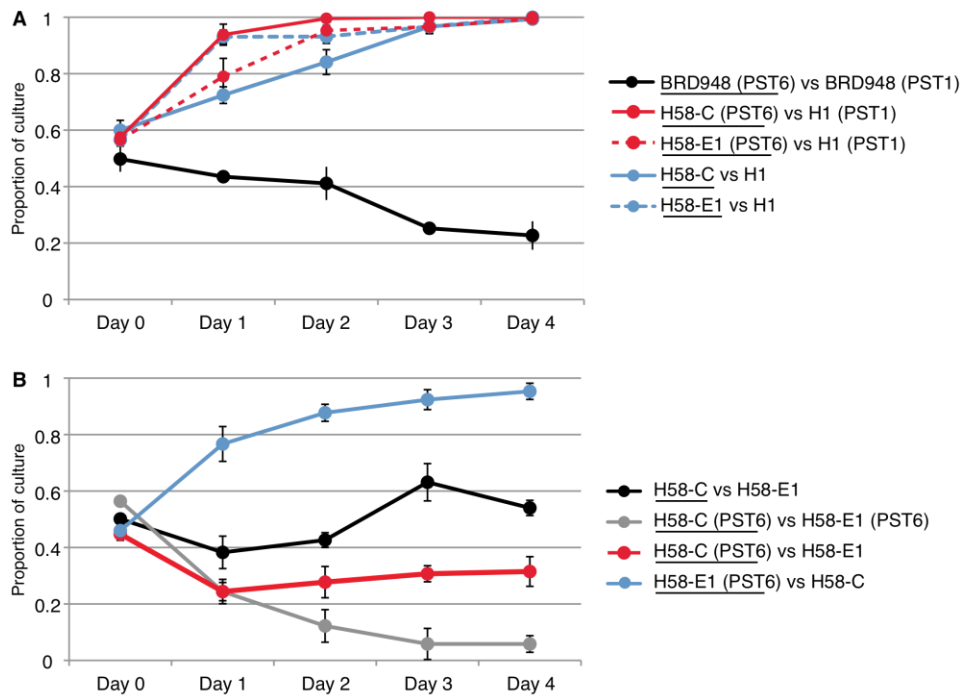


Figure 4. Competitive growth assays *S. Typhi* H58 and H1 with and without IncHI1 plasmids

The dynamics of three competitive growth assays conducted over four days of sequential sub-culture. (A) Competition between PST1 and PST6 plasmid-bearing *S. Typhi*, including competition in a common strain background (attenuated laboratory strain *S. Typhi* BRD948; haplotype H10) and wildtype *S. Typhi* haplotype-strain combinations. (B) Competition between wildtype *S. Typhi* H58 strains with and without PST6 plasmids. For BRD948 assays, the proportion of PST1- and PST6-bearing bacteria at each time point was calculated by streaking an aliquot of the sample onto agar plates and testing random colonies using a PCR that differentiates PST1 and PST6. For *S. Typhi* haplotype H58 vs H1 assays, the proportion of H58 and H1 chromosomes at each time point was calculated by quantifying the relative abundance of two alleles at a SNP locus that differs between H58 and H1 *S. Typhi*,

using quantitative PCR. For all assays, experiments were replicated 4-5 times; data points represent the mean proportion of the PST6-bearing strain (underlined in the legend) and error bars show the standard deviation of the proportion of the PST6-bearing strain.

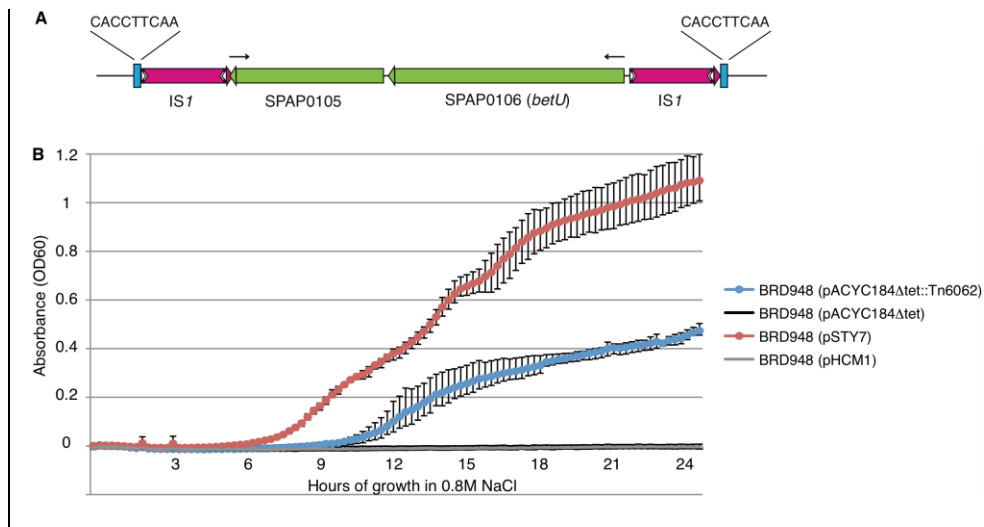


Figure 5. Tn6065 and its effect on osmotolerance of *S. Typhi* BRD948

(A) Schematic diagram of Tn6062 showing the two flanking IS1 and the internal CDSs (based on nucleotide sequence of pAKU_1). The two black arrows represent primers used to clone the putative transporters to pACYC184.

(b) Growth curves showing the osmotolerance of BRD948 (pSTY7; PST6) and BRD948 (pACYC184Δtet::Tn6062) in 0.8M NaCl LB broth. Error bars indicate range of maximum and minimum values.

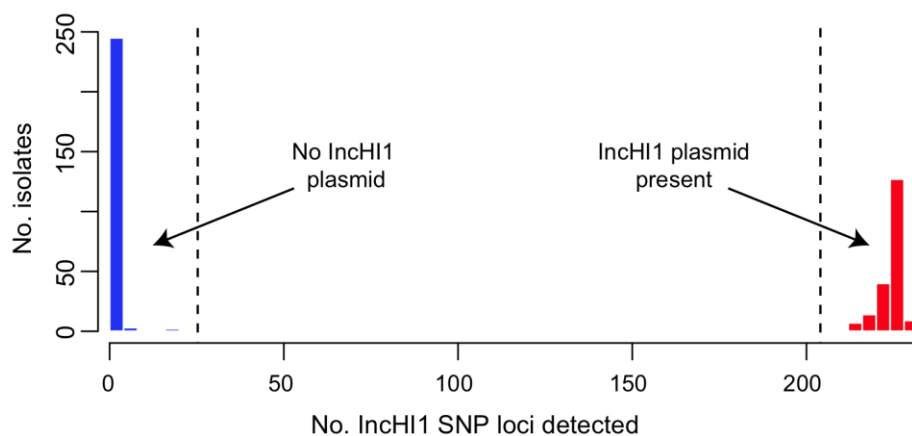


Figure S1. Distribution of IncHI1 loci among *S. Typhi* isolates. X-axis indicates the number of IncHI1 plasmid loci (out of 231 targets) generating a fluorescent signal in the Illumina GoldenGate assay. Isolates clearly fall into two groups: either >90% of IncHI1 target loci were detected, indicating presence of an IncHI1 plasmid (red) or <10% of IncHI1 target loci were detected, indicating absence of any IncHI1 plasmid (blue).

Supplementary Information

Table S1 – Bacterial isolates analysed in this study

Table S2 – IncHI1 SNP loci targeted in this study

Table S3 – Phenotype array results

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