

Isolation of *Salmonella enterica* subspecies *enterica* serovar Paratyphi B dT⁺, or *Salmonella* Java, from Indonesia and alteration of the *d*-tartrate fermentation phenotype by disrupting the ORF STM 3356

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Salmonella enterica subspecies *enterica* serovar Paratyphi B [O1,4,(5),12:Hb:1,2] can cause either an enteric fever (paratyphoid fever) or self-limiting gastroenteritis in humans. The *d*-tartrate non-fermenting variant *S. enterica* subsp. *enterica* serovar Paratyphi B dT[−] (*S. Paratyphi* B) is the causative agent of paratyphoid fever, and the *d*-tartrate fermenting variant *S. enterica* subsp. *enterica* serovar Paratyphi B dT⁺ (*S. Paratyphi* B dT⁺; formerly called *Salmonella* Java) causes gastroenteritis. *S. Java* is currently recognized as an emerging problem worldwide. Twelve dT⁺ *S. Java* isolates were collected in Indonesia between 2000 and 2002. One-third of them contained *Salmonella* genomic island 1 (SGI1), which gives the multidrug-resistant phenotype to the bacteria. In this study, a PCR-based method to detect a single nucleotide difference responsible for the inability to ferment *d*-tartrate, reported elsewhere, was validated. The *d*-tartrate fermenting phenotype of *S. Java* was converted to the non-fermenting phenotype by the disruption of the ORF STM 3356, and the *d*-tartrate non-fermenting phenotype of the ORF STM 3356-disrupted strain and the dT[−] reference strain was changed to the dT⁺ phenotype by complementing ORF STM 3356 *in trans*. The results show that the dT⁺ phenotype requires a functional product encoded by STM 3356, and support the use of the PCR-based discrimination method for *S. Paratyphi* B and *S. Java* as the standard differentiation method.

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INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Paratyphi B, antigenic formula O1,4,(5),12:Hb:1,2, is one of the causative agents of enteric fever, along with *Salmonella*

Typhi, and *Salmonella* Paratyphi A and C. It has been recognized, however, that this serovar, as well as causing systemic disease, can cause a less severe gastroenteritis in humans. The fermentation of dextrorotatory [(L(+))]-tartrate (*d*-tartrate) has been used to differentiate between the *d*-tartrate non-fermenting *S. enterica* subsp. *enterica* serovar Paratyphi B dT[−] (*S. Paratyphi* B) and the *d*-tartrate fermenting *S. enterica* subsp. *enterica* serovar Paratyphi B dT⁺ (*Salmonella* Java). *S. Paratyphi* B causes systemic

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Abbreviations: MDR, multidrug-resistant; MLST, multilocus sequence typing; SGI1, *Salmonella* genomic island 1.

disease (paratyphoid fever) in humans and *S. Java* triggers gastroenteritis in humans; however, the latter has also been isolated from animals (Barker, 1985; Chart, 2003). Multidrug-resistant (MDR) *S. Java* strains have been an emerging problem since the 1990s (Miko *et al.*, 2002), and recent reports have shown that the incidence of *S. Java* is increasing significantly in a number of European countries, Canada and Malaysia (Goh *et al.*, 2003; Miko *et al.*, 2002; Mulvey *et al.*, 2004; Threlfall *et al.*, 2005; Weill *et al.*, 2005). The MDR strains are generally resistant to ampicillin, chloramphenicol, streptomycin/spectinomycin, sulfonamides and tetracycline (ACSSuT), and the genes conferring antimicrobial resistance are located on a 14 kb region at the 3' end of a chromosomal genomic island known as *Salmonella* genomic island 1 (SGI1). The 43 kb SGI1 was first identified in *Salmonella* Typhimurium DT104 (Boyd *et al.*, 2001), and has also been found in other serovars, i.e. *Salmonella* Agona, *S. Paratyphi* B, *Salmonella* Albany and *Salmonella* Newport (Doublet *et al.*, 2003, 2004; Meunier *et al.*, 2002). SGI1 harbours genes responsible for pentaresistance and is inserted into the chromosome between the *thdF* and *int2* genes in *S. Typhimurium* and between the *thdF* and *yidY* genes in other serovars (Doublet *et al.*, 2004). Recently, SGI1 has been characterized as an integrative mobilizable element which can be conjugally transferred to *S. enterica* and *Escherichia coli* (Doublet *et al.*, 2005). Twelve *S. Java* isolates were collected in Indonesia between 2000 and 2002. Five isolates showed resistance to more than two antibiotics and three of those contained SGI1. One isolate contained a variant of SGI1, SGI1-C, responsible for resistance to streptomycin and sulfonamide. One of the isolates was resistant to streptomycin and chloramphenicol but did not contain the SGI1.

The emergence of the *S. Java* strains as a public health problem calls for efficient identification and detection of this serovar. The discrimination method using lead acetate for *d*-tartrate fermentation takes up to 6 days and can give unreliable results (Barker, 1985; Malorny *et al.*, 2003). A PCR-based discrimination method has been developed by Malorny *et al.* (2003) and tested empirically against a well-defined collection of dT^+ and dT^- *Salmonella* strains. Among the ORFs possibly involved in *d*-tartrate fermentation, a single nucleotide difference in the ORF STM 3356 (a putative cation transporter) between *d*-tartrate fermenting and non-fermenting strains has been recognized. In dT^+ strains, an ATG translation start codon is present for STM 3356, whereas this is altered to ATA in dT^- strains. A discriminatory PCR primer using this one-nucleotide difference gave 100% discrimination of dT^+ and dT^- *Salmonella* strains. Although the PCR-based discrimination method gives reliable results, it has not been elucidated that the encoded product of STM 3356 is compulsory for the *d*-tartrate fermenting phenotype. To determine whether the product encoded by STM 3356 is essential for the *d*-tartrate fermenting phenotype, we disrupted the STM 3356 ORF by inserting a suicide vector in the middle of the STM 3356 ORF in a *d*-tartrate fermenting *S. Java* isolate. The STM 3356

mutant showed a *d*-tartrate non-fermenting phenotype, while the dT^- phenotype of the STM 3356 mutant and the dT^- reference strain NCTC 3176 was changed to a dT^+ phenotype by complementing with the STM 3356 of a dT^+ strain *in trans*, which implies that the functional product encoded by STM 3356 is required for the *d*-tartrate fermenting phenotype. These results support the use of the PCR-based discrimination method of Malorny *et al.* (2003) as the standard method for identification of *d*-tartrate fermenting and non-fermenting *S. Paratyphi* B strains. This is particularly important in view of the increased incidence of *S. Java* infections around the world.

METHODS

Bacterial strains and serotyping. During a population-based typhoid fever surveillance study at Jakarta, Indonesia, between 2000 and 2002, twelve *Salmonella* group B isolates were collected and transported to the International Vaccine Institute (IVI), Seoul, Korea, for phenotypic and genotypic analyses. All isolates were serotyped by the Kauffmann–White scheme (Kauffmann, 1955), resulting in the antigenic formula O1,4,(5),12:Hb:1,2 specific to *S. Paratyphi* B, or *S. Java*. The *d*-tartrate fermentation test reference strains NCTC 3176 and NCTC 5706 were purchased from the UK National Collection of Type Cultures (NCTC).

Antimicrobial susceptibility tests. All isolates were screened for resistance to 20 antimicrobial agents by either the disc diffusion method (BBL, Sensi-Disc, Becton Dickinson) or the Vitek automated system and susceptibility testing card GNS-433 (bioMérieux). The following antibiotic agents were tested: ampicillin, nalidixic acid, trimethoprim/sulfamethoxazole (SXT), streptomycin, ciprofloxacin, chloramphenicol, tetracycline, amikacin, ampicillin/sulbactam, aztreonam, ceftazidime, cefepime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, piperacillin/tazobactam, sulfonamides and tobramycin. MICs of the antibiotics to which the isolates were resistant were determined by the agar dilution method. *E. coli* ATCC 25922 was used as a quality reference for all the tests.

Multilocus sequence typing (MLST) and genetic analyses. All isolates were grown overnight on tryptic soy agar (Difco) plates at 37 °C. The genomic DNA was prepared from a bacterial culture plate using a Prepman Ultra kit (Applied Biosystems). The seven genes for the MLST analysis, *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *hisD* (histidinol dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (α -ketoglutarate dehydrogenase) and *thrA* (aspartokinase + homoserine dehydrogenase), were amplified using the primers and PCR conditions described elsewhere (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica/documents/primersEnterica.html>). The purified PCR products were sequenced in both directions by using a BigDye cycle sequencing kit (ABI). Sequencing was performed on an ABI 3770 automatic sequencer. Alleles were assigned by comparing the sequences to those in the *Salmonella* MLST database. No new allele type was identified, and those already present were assigned the relevant allele number. The presence of SGI1 was confirmed by PCR amplification of left and right junctions, as described elsewhere (Weill *et al.*, 2005). In addition to that of either junction, the presence of SGI1 was further confirmed by PCR mapping of two different regions on SGI1. A primer pair P1f (5'-ATA ACG GCA GGT TCC GGT TC-3')/P1r (5'-CCG ATG AAG CGC ACA AAT TTG-3') was used to amplify the p1-9 probe region (from nucleotide 20173 to 21109), and the presence of the QS probe region was verified by PCR amplification using the primer pair QS1 (5'-ATG AAA GGC TGG CTT TTT CTT G-3')/

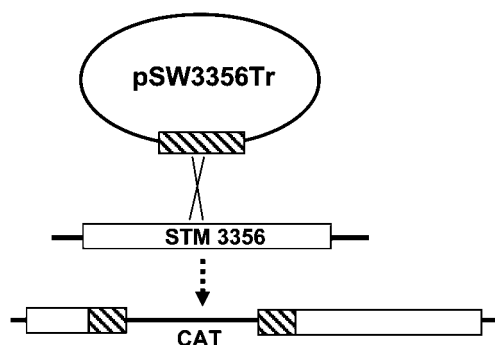


Fig. 1. Construction of STM 3356 mutant strain. The recombinant suicide plasmid pSW3356Tr containing an internal 300 nt fragment of STM 3356 (hatched box) was transferred by conjugation into IB75. The streptomycin/chloramphenicol resistant IB75*ins* mutant resulting from the integration of pSW3356Tr was verified by PCR, as described in Methods. CAT, chloramphenicol acetyl transferase.

QS2 (5'-TGA GTG CAT AAC CAC CAG CC-3') (Weill *et al.*, 2005). Two loci, *sopE1* and *avrA*, were amplified by PCR to distinguish between strains with systemic and enteric outcomes of disease, as described elsewhere (Prager *et al.*, 2003).

STM 3356 mutant construction and *trans*-complementation.

A streptomycin/sulfonamide-resistant isolate, IB75, was used for the construction of an STM 3356 mutant to facilitate counter-selection during the conjugal transfer of a suicide plasmid and *trans*-complementation. The ORF STM 3356 in IB75 was disrupted by insertion of a recombinant suicide plasmid. The internal 300 nt DNA fragment of STM 3356 (from nucleotide 101 to 400) was amplified with primers Bam3356 (5'-CCG GGA TCC TGG CCT TAG TTA TCA CCG-3') and Eco3356 (5'-CGG GAA TTC GAC GAG AAC GCG AGA AGC-3') using PCR. This fragment was inserted into a suicide plasmid pSW23.oriT (Kim *et al.*, 2005), giving pSW3356Tr. The recombinant plasmid was transferred by conjugation to IB75 (Fig. 1). In the IB75*ins* mutant, the STM 3356 gene was split into two truncated fragments, a 5' 400 bp fragment and a 3' 1169 bp fragment (out of 1269 nt of STM 3356). The disruption of STM 3356 was confirmed by PCR using STM5 (5'-CTG CAT CTT TCC TGA GAA AC-3')/SW23.5 (5'-TGC TCT GCG AGG CTG GCC GGC-3'), and STM3 (5'-TCA GAA CAG CTT CCG TTA AT-3')/SW23.3 (5'-CAC AGG AAC ACT TAA CGG CTG-3') primer pairs.

To construct a *trans*-complementing plasmid, a DNA fragment encompassing the 5' upstream region of STM 3356 and the STM 3356 ORF (from the termination codon of STM 3357 to the termination codon of STM 3356) was amplified by PCR with primer set Bam5 (5'-GCG GCC GGA TCC TCT TTT CGC CTG CGT GAG CAT CGC-3')/Hin3 (5'-CGC GGG AAG CTT TCA GTG GTG GTG GTG GTG CGG ATG AAA AGG GAA AAG GAT TGG-3') and inserted into pACYC184. The resulting plasmid, pAC3356, was used to transform the IB75*ins* mutant and the *d*-tartrate fermentation test dT⁻ reference strain NCTC 3176. Each *trans*-complemented strain was subjected to a lead acetate test to demonstrate the restoration of the *d*-tartrate fermentation phenotype.

Lead acetate protocols. A lead acetate test was performed as described elsewhere, with slight modifications (Malorny *et al.*, 2003). Briefly, 10 g l⁻¹ Bacto Peptone (Difco) was autoclaved at 121 °C for 15 min. Potassium sodium tartrate tetrahydrate (Sigma) was added to a final concentration of 1 %. The pH was adjusted with NaOH to

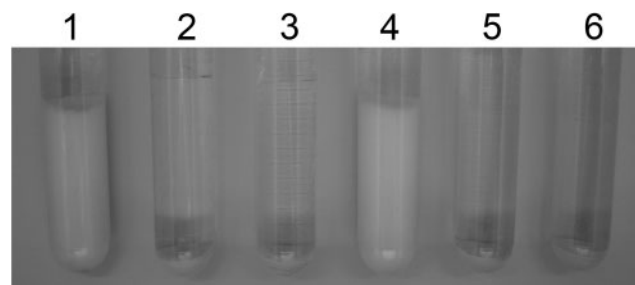


Fig. 2. Lead acetate test of *S. Paratyphi B* strains. A fine fluffy precipitate (tubes 1 and 4) indicates the *d*-tartrate non-fermenting phenotype, and a small precipitate (tubes 2, 3, 5 and 6) indicates the *d*-tartrate fermenting phenotype. Tubes: 1, dT⁻ reference strain NCTC 3176; 2, dT⁺ reference strain NCTC 5706; 3, IB75; 4, IB75*ins*; 5, IB75*ins*/pAC3356 *trans*-complemented strain; 6, NCTC 3176/pAC3356 *trans*-complemented strain.

7.4. Bromothymol blue sodium salt (Sigma) was added to a final concentration of 0.0023 % as an indicator. The broth was sterilized two times for 15 min at 100 °C. The broth was dispensed in 8 ml aliquots into sterilized round-bottomed test tubes. Approximately 5 × 10⁷ c.f.u. of each bacterium (dT⁻ reference strain NCTC 3176, dT⁺ reference strain NCTC 5706, IB75, IB75*ins* mutant and two *trans*-complemented strains) was inoculated into each tube. The cultures were incubated at 37 °C for 3 and 6 days aerobically without shaking. After incubation, the cultures were tested for *d*-tartrate utilization by the addition of a saturated aqueous lead acetate solution (Merck). The immediately resulting precipitate was homogenized by brief mixing. dT⁺ strains were detected by the formation of a small precipitate after the lead acetate addition, while the formation of a fluffy fine precipitate after the lead acetate addition indicated a dT⁻ strain (Fig. 2) (Malorny *et al.*, 2003).

RESULTS AND DISCUSSION

Identification of *S. Paratyphi B* dT⁺, or *S. Java*

Twelve *S. Java* isolates collected from patients' stool samples in Indonesia were found to have the antigenic formula O1,4,(5),12:Hb:1,2, specific for *S. Paratyphi B*, or *S. Java*, by standard tests (isolate numbers IB70 to IB81). Lead acetate results showed that all isolates were dT⁺, and PCR using primers 166/167 and sequence analysis of the STM 3356 amplified fragment supported the *d*-tartrate positive phenotype, as described elsewhere (Malorny *et al.*, 2003). All isolates were subjected to MLST analysis using seven genetic loci, and their allele types were all identical as *aroC*: 2, *dnaN*: 14, *hemD*: 24, *hisD*: 14, *purE*: 2, *sucA*: 19 and *thrA*: 8, which correspond to sequence type (ST) 43. ST 43 has previously been reported as *S. Paratyphi B* var. *Java* from Denmark (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica>). The virulence genes *sopE1* and *avrA*, which are usually found in systemic and enteric pathovars, respectively (Prager *et al.*, 2003), were not present among the isolates (data not shown). From these results, 12 isolates were identified as *S. Paratyphi B* dT⁺, or *S. Java*.

Table 1. Antibiotic resistance and the presence of SGI1 in *S. Paratyphi* B dT⁺ isolates in this study

Isolate	Antimicrobial susceptibility test*						SGI1 junction PCR			SGI1 variant
	Amp	Tet	Nal	Str	Chl	MDR profile†	Left junction (<i>thdF</i> -S001)	Right junction (S044- <i>int2</i>)	Right junction (S044- <i>yidY</i>)	
IB70	≥256‡	128	4	256	≥256	ACSSuT	+	–	+	SGI1
IB71	1	8	8	16	4		–	–	–	
IB72	1	8	4	128	128	CS	–	–	–	
IB73	2	8	8	16	4		–	–	–	
IB74	1	8	4	16	4		–	–	–	SGI1-C
IB75	2	8	8	128	4	SSu	+	–	+	
IB76	≥256	128	8	128	≥256	ACSSuT	+	–	+	
IB77	2	8	8	8	4		–	–	–	
IB78	2	8	4	8	4		–	–	–	SGI1
IB79	2	8	8	8	4		–	–	–	
IB80	≥256	128	8	128	≥256	ACSSuT	+	–	+	
IB81	2	8	8	8	4		–	–	–	

*Amp, ampicillin; Tet, tetracycline; Nal, nalidixic acid; Str, streptomycin; Chl, chloramphenicol.

†A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamide; T, tetracycline. Sulfonamide resistance was tested on LB plates containing 100 µg sulfonamide ml⁻¹.

‡MIC by agar dilution test.

Antimicrobial resistance and the presence of SGI1

Of 12 isolates examined for antibiotic susceptibility, five (IB70, IB72, IB75, IB76 and IB80) were resistant to at least two antibiotic agents, and seven were sensitive to all the antibiotic agents tested (Table 1). All of the isolates were sensitive or intermediate in resistance to nalidixic acid. Three isolates, IB70, IB76 and IB80, showed a pentaresistant phenotype – resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (ACSSuT) – a common characteristic of SGI1. IB75 was resistant to streptomycin and sulfonamides, and IB72 was resistant to streptomycin and chloramphenicol. To confirm the presence of SGI1 in the isolates, PCR amplification of the left and right junctions and two internal regions, the p1-9 probe region and the QS probe region, of SGI1 was performed as described elsewhere (Boyd *et al.*, 2001, 2002). Four isolates (IB70, IB75, IB76 and IB80) were positive for the left junction (*thdF*-S001) and right junction (S044-*yidY*), and two internal regions of SGI1. No DNA was amplified with the PCR primer set for the S044-*int2* fragment, which implies the absence of the *int2*-retron sequence, as described elsewhere (Meunier *et al.*, 2002). This is consistent with other reports that the SGI1 is located between the *thdF* and *int2* genes in serovar *S. Typhimurium*, while it is located between the *thdF* and *yidY* genes in other serovars (Boyd *et al.*, 2001; Doublet *et al.*, 2004). According to the antibiotic-resistance phenotype and the PCR-mapping results, IB70, IB76 and IB80 contain SGI1, and IB75 contains a variant of SGI1, SGI1-C (Boyd *et al.*, 2002). Although IB72 is resistant to streptomycin and chloramphenicol, no SGI1-specific fragment was amplified, which

suggests that this isolate has acquired the resistance mechanism independently from SGI1. *S. Java* isolates carrying SGI1 were detected at 33% (four of 12) in this study, including one isolate carrying SGI1-C, a variant of SGI1. Ribotyping results obtained after *EcoRI* digestion using the RiboPrinter automated system (DuPont) revealed that all the isolates belonged to the same ribotype (data not shown). From the results that show that all the isolates have the same MLST sequence type and ribotype, the *S. Java* isolates examined in this study appear to be closely related. However, for further detailed studies, including the distribution, occurrence and phylogenetic relationship among the isolates in Indonesia and adjacent areas, a larger collection of isolates, and subtyping methods such as PFGE and IS200 analysis, should be performed. Miko *et al.* (2002) have described that from the mid-1990s, new clones of *S. Java* emerged, and one clonal lineage displaced the older ones in Germany and neighbouring countries. Goh *et al.* (2003) have reported that one major cluster of *S. Java* was observed among Malaysian isolates. The relationship of *S. Java* strains among the European and non-European lineages remains to be studied.

d-Tartrate fermenting phenotype change by the disruption of ORF STM 3356

To determine whether the product of STM 3356 is essential for *d*-tartrate fermentation, we constructed a derivative of the dT⁺ strain IB75, in which the STM 3356 was inactivated by insertion of an integrative plasmid carrying an internal fragment of STM 3356 (IB75*ins*). The STM 3356 mutant exhibited a *d*-tartrate non-fermenting phenotype (Fig. 2). The number of bacteria in the inocula did not affect the

results within the range 5×10^9 to 5×10^7 bacteria. These results indicate that the product of ORF STM 3356 is necessary for *d*-tartrate fermentation. To determine whether expression of wild-type STM 3356 would be sufficient to revert the negative phenotype of the IB75*ins* mutant and *d*-tartrate non-fermenting reference strain NCTC 3176, we constructed the plasmid pAC3356, a derivative of pACYC184 carrying a DNA fragment encompassing the upstream region of STM 3356 (up to the termination codon of upstream ORF STM 3357) and the whole STM 3356 ORF from the dT⁺ reference strain NCTC 5706. For the detection of the expression of STM 3356 from pAC3356, we included six His residues at the end of ORF 3356 on pAC3356. The derivative of NCTC 3176 harbouring pAC3356 exhibited a *d*-tartrate fermentation-positive phenotype (Fig. 2), although the expression of STM 3356 was not detected by Western blot using Penta-His Antibody (Qiagen; data not shown). This result indicates that the negative phenotype of NCTC 3176 is solely due to the lack of production of STM 3356. The dT⁻ phenotype of IB75*ins* was also converted into the dT⁺ phenotype by the same *trans*-complementing plasmid (Fig. 2), and the complementing plasmid was maintained for several overnight culture passages without a selection marker in both *trans*-complemented bacteria.

In conclusion, we have found that *S. Java* strains harbouring the SGI1 element are common in Indonesia (four of 12 isolates), although we need to analyse a larger number of strains for more precise results. Since the SGI1 element can be transferred to other *Salmonella* serovars (Doublet *et al.*, 2005), it is important to monitor the dissemination of the SGI1 element, especially to *S. Typhi* and *S. Paratyphi A*, which are common in this area (Tjaniadi *et al.*, 2003). We have shown the involvement of the functional product of STM 3356 in *d*-tartrate fermentation, and our results strongly support the effectiveness of the method using specific PCR for *d*-tartrate fermentation phenotype differentiation suggested by Malorny *et al.* (2003), although the exact role of the protein remains to be revealed.

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