The artAB genes encode a putative ADP-ribosyltransferase toxin homologue associated with Salmonella enterica serovar Typhimurium DT104

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Many bacterial pathogens encode ADP-ribosyltransferase toxins. The authors identified an ADP-ribosyltransferase toxin homologue (ArtA, ArtB) in Salmonella enterica serovar Typhimurium (S. typhimurium) DT104. ArtA is most homologous to a putative pertussis-like toxin subunit present in Salmonella typhi (STY1890) and Salmonella paratyphi A (SPA1609), while ArtB shows homology to a hypothetical periplasmic protein of S. typhi (STY1364) and S. paratyphi A (SPA1188), and a putative pertussis-like toxin subunit in S. typhi (STY1891) and S. paratyphi A (SPA1610). The artA gene was detected from the phage particle fraction upon mitomycin C induction, and the flanking region of artAB contains a prophage-like sequence, suggesting that these putative toxin genes reside within a prophage. Southern blotting analysis revealed that artA is conserved in 12 confirmed DT104 strains and in four related strains which are not phage-typed but are classified into the same group as DT104 by both amplified-fragment length polymorphism and pulsed-field gel electrophoresis. Except for one strain, NCTC 73, all 13 S. typhimurium strains which were classified into different groups from that of DT104 lacked the artA locus. The results suggest that phage-mediated recombination has resulted in the acquisition of art genes in S. typhimurium DT104 strains.

INTRODUCTION

Salmonella enterica serovar Typhimurium (S. typhimurium) is a common cause of salmonellosis in humans and animals. Phage typing is a commonly used method that has proved useful in epidemiological surveillance of S. typhimurium infection. The Anderson phage-typing scheme currently in use distinguishes 207 definitive types (DTs) with 34 phages (Anderson et al., 1977). In recent years, a multidrug-resistant S. typhimurium DT104 has emerged and has spread throughout many countries (Glynn et al., 1998; Sameshima et al., 2000; Threlfall et al., 1994, 1996; Villar et al., 1999). The organism has a core pattern of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (Briggs & Fratamico, 1999; Ridley & Threlfall, 1998), and this resistance is encoded by a chromosomally located locus containing class 1 integron structures (Arcangioli et al., 1999; Ng et al., 1999; Sandvang et al., 1998). Although the occurrence of S. typhimurium DT104 in humans and domestic animals is increasing, an enhanced virulence-associated phenotype of this organism has not yet been detected. However, it has been postulated that, in addition to its multiresistance, novel virulence mechanisms may have evolved in DT104, which would account for its increased incidence (Glynn et al., 1998). Recently, we isolated a temperate phage, ST104, which is common among DT104 strains (Tanaka et al., 2004). The complete sequence of the...
ST104 genome exhibits high similarity to that of P22 and a gene encoding a possible virulence factor has not yet been identified in the genome of ST104 (Tanaka et al., 2004).

In the present study, we have identified another prophage-like element in the supernatant of a S. typhimurium DT104 isolate treated with mitomycin C. Genome walking analysis revealed the presence of putative pertussis-toxin genes in the flanking region of the prophage-like element. Here we show the distribution of this novel gene among S. typhimurium DT104 isolates.

METHODOLOGY

Bacterial strains and plasmid. Thirty strains of S. typhimurium, including 12 previously described DT104 strains (Tanada et al., 2001) were examined. Escherichia coli XL-1 Blue and pBluescript II (Stratagene) were used in the cloning experiment. Bacteria were cultured in Luria–Bertani (LB) broth or on LB agar.

Induction of lysogen. In order to identify putative lysogenic phages, the S. typhimurium DT104 strain U1 was grown overnight in 10 ml LB broth, before subculturing at 1:10 into 50 ml culture with shaking. After the early exponential phase, mitomycin C was added at a concentration of 0.5 μg ml⁻¹ and the mixture incubated for a further 16 h with vigorous shaking as previously described (Tanaka et al., 2004; Yee et al., 1993). The bacterial cells were removed by centrifugation (9800 g, 10 min) and filtered through a 0.45 μm membrane filter. After digesting bacterial DNAs and RNAs by DNase (100 μg ml⁻¹) and RNase (50 μg ml⁻¹) for 2 h at 37 °C, phage particles were precipitated with 10% (w/v) PEG 6000 and 1 M NaCl following the methods described by Ikebe et al. (2002). DNAs in the particles were then extracted by phenol and precipitated with ethanol.

Cloning and DNA sequencing. DNA sequencing was performed with an automated DNA sequencer (ABI Prism 377; Perkin-Elmer Applied Biosystems) by using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems). The 0.4 kb HindIII fragment of a putative phage genome was cloned in pBluescript II and the initial sequence information of the clone was obtained with universal and reverse primers for pUC/M13 vectors. This sequence was used to create customized oligonucleotides for primer walking.

Then adjacent DNA sequences upstream and downstream of the 0.4 kb HindIII fragment in the U1 genome were investigated with a Clontech Universal Genome Walker Kit. Details of the methodology used can be found in the user manual at http://www.clontech.com/. Briefly, U1 genomic DNA was digested with one of four different blunt-ended restriction enzymes provided by the manufacturer. The purified restricted DNA fragments were ligated to an adaptor supplied by the manufacturer and became a part of four genome-walking libraries generated by the four different restriction enzymes. Specific primers based on the sequence of the 0.4 kb HindIII fragment were designed and used in combination with adaptor-specific primers to amplify the large genomic segments adjacent to the 0.4 kb HindIII fragment by long-distance PCR using a Takara LA PCR Kit in a 9700 thermal cycler (Applied Biosystems) as described by the manufacturer. PCR products were purified with a Qiagen QIAquick PCR Purification Kit for sequencing.

PCR. Standard PCR was carried out in a mixture of 50 μl containing 1 x PCR buffer with 0.2 mM MgCl₂, 2.5 U AmpliTaq DNA polymerase, 0.2 mM deoxynucleotide triphosphates, 0.5 μM primers and 50 ng genomic DNA. PCR amplification was performed with the first denaturation cycle at 95 °C for 1 min, followed by 30 cycles, each consisting of 30 s denaturation at 94 °C and 30 s annealing at 55 °C, and 1 min extension at 72 °C. Incubation for 3 min at 72 °C followed to complete extension.

Southern hybridization analysis. A DNA fragment was amplified with the specific primer set ART-1 (5’-CTGGTTATGCAAGTGCTGT-3’) and ART-2 (5’-CTCCCGTGCGTCATAAAAAC-3’), which amplified a 566 bp internal fragment of artA gene. The PCR product was purified with a QiAquick Gel Extraction Kit (Qiagen) and was labelled with digoxigenin (DIG)-11-dUTP by random priming using a DIG High Prime Labelling Kit (Roche Diagnostics) as described by the manufacturer. Genomic DNA was prepared from S. typhimurium strains by the method previously described (Tamada et al., 2001) and digested with HindIII, separated on 0.8% (w/v) agarose gel and transferred to a positive membrane (Roche Diagnostics) by the capillary method. Prehybridization (>30 min) and hybridization (>16 h) using Easy Hyb solution (Roche Diagnostics) under high-stringency conditions and digoxigenin detection of hybrids were carried out using a DIG Luminescent Detection Kit (Roche Diagnostics), following the manufacturer’s instructions. Hyper MP film (Amersham International) was exposed to membranes for 1–10 min at room temperature and developed in a Kodak X-Omat processor. PFGE of DNA extracted from the phage particle fraction was performed using 1·2% (w/v) agarose gel and a 2 s switching interval was applied for 10 h. Subsequent transfer of DNAs and Southern hybridization were carried out as described above.

Fig. 1. HindIII digests of the phage DNA fraction induced by mitomycin C treatment in S. typhimurium DT104 strain U1. M, molecular mass standards; A, ST104; B, phage DNA fraction induced by mitomycin C.
RESULTS

Identification of putative pertussis-like toxin genes

Induction of putative lysogenic phages from the DT104 strain U1 with mitomycin C was carried out and DNA extracted from these particles was digested with HindIII. The restriction digestion pattern of the DNA extract was different from that of phage ST104, which is common in DT104 (Tanaka et al., 2004) (Fig. 1). This result indicates that DT104 has another prophage in addition to ST104. In order to examine the prophages in DT104, fragments of the DNA extract digested with HindIII were cloned. The 0.4 kb fragment was cloned and sequence analysis was carried out. DNA sequencing of the fragment revealed a partial ORF (ORF1) which showed homology to a hypothetical protein (sb48) encoded by the temperate phage ST64B of S. typhimurium DT64 (Table 1, Fig. 2). Genomic walking was then used to sequence 202 bp and 3620 bp in the regions upstream and downstream of the sequence of the 0.4 kb HindIII fragment, respectively. The position and orientation of the predicted ORFs are shown in Fig. 2. Seven complete or partial ORFs were identified. BLASTP searches of the GenBank and EMBL databases were carried

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<th>Function</th>
<th>Length of homologue (aa)</th>
<th>Homologous residues (%)</th>
<th>Identity (%)</th>
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out using deduced amino acid sequences as query sequences (Table 1).

ORF2, just downstream of ORF1, is predicted to express a protein with homology to a conserved hypothetical protein of *Shigella flexneri* 2a, a hypothetical protein of *Salmonella typhi* (STY2050) and an unknown protein encoded by prophage CP-9330 of *E. coli* O157:H7. Downstream of ORF2 we detected two ORFs (designated ORF3 and ORF4) encoding polypeptides of 241 and 141 aa with predicted molecular masses of 27,592 and 15,823, respectively. There are 132 bases between these ORFs. The translation start codons of both ORF3 and ORF4 are preceded by a potential ribosome-binding site located 7 bp before the initiation codon. The deduced amino acid sequences of both ORF3 and ORF4 were preceded by an N-terminal sequence with the typical features of a signal peptide composed of 18 and 21 aa, respectively. Database searches revealed that the predicted amino acid sequence encoded by the larger ORF3 shared 59 % identity in a 240 aa overlap to the putative pertussis-like toxin subunit in *S. typhi* (STY1890) and in *Salmonella paratyphi* A (SPA1609), and 33 % identity in a 196 aa overlap to the precursor of the pertussis toxin S1 subunit (PtxA), which is an ADP-ribosyltransferase toxin (Table 1, Fig. 3). The deduced polypeptide encoded by ORF4 revealed homology to a hypothetical periplasmic protein of *S. typhi* (STY1364), and of *S. paratyphi* A (SPA1188) (73 % identity in a 141 aa overlap). This product also showed homology to a putative pertussis-like toxin subunit of *S. typhi* (STY1891) and *S. paratyphi* A (SPA1610) (30 % identity in a 111 aa overlap), and a putative exported protein of *Yersinia pestis* (YP00337) (29 % identity in a 96 aa overlap) (Table 1, Fig. 4). The products of STY1891 and SPA1610 are similar to pertussis toxin subunit 2 and 3 precursors (PtxB), with 30-7 % identity in a 101 aa overlap and 29-6 % identity in a 108 aa overlap, respectively (Parkhill et al., 2001). Therefore, ORF3 and ORF4 are designated *artA* and *artB* (ADP-ribosyltransferase toxin homologue), respectively.

The derived amino acid sequences of ORFs 5–7 show similarity to putative prophage proteins of *S. typhi* (STY1040, STY1041 and STY1042, respectively). Partial ORF7 is also similar to the lytic enzyme of phage ST64B, which is a temperate phage of *S. typhimurium* DT64.

### Presence of *artA* sequence among *S. typhimurium* strains

In order to examine the presence of the *artA* sequence among *S. typhimurium* isolates, Southern hybridization was performed. A PCR fragment specific to the coding sequence of *artA* was labelled and used as a probe. *HindIII*-digested chromosomal DNA from all 12 strains of DT104, and four strains which are not phage-typed but were classified into the same group as DT104 by PFGE and amplified-fragment length polymorphism (AFLP) analysis (Tamada et al., 2001), revealed hybridizing signals at 2-2 kb (Fig. 5). The same signal was also detected in NCTC 73, a strain isolated from humans in France in 1917, which is classified into a different group from that of DT104 (Tamada et al., 2001). No intense signals were detected following hybridization of the *artA* probe with DNA from the 13 other *S. typhi*-murium strains which belonged to groups other than DT104 (Tamada et al., 2001).
Induction of temperate phages by mitomycin C treatment

Since a phage sequence was found in the flanking region of artAB, we examined the inducibility of prophage in strain U1 by using mitomycin C. When cells were induced, a 42 kb DNA fragment, which is the same size as ST104, became detectable from the phage particle fraction by ethidium bromide staining (Fig. 6, lane 2) and this band reacted with an artA probe (Fig. 6, lane 4). A low level of induction without inducer was detected (Fig. 6, lane 3).

DISCUSSION

In the present study we have shown the presence of ORFs similar to ADP-ribosyltransferase toxin genes in S. typhimurium DT104. Recent genomic analysis of S. typhi CT18 and S. typhimurium LT2 has revealed the presence of ORFs encoding proteins homologous to subunits of pertussis toxin in S. typhi CT18, which were apparently absent in S. typhimurium strain LT2 (McClelland et al., 2001; Parkhill et al., 2001). However, it is not yet known whether such a putative toxin of S. typhi is actually expressed and active (Pallen et al., 2001). A homologue of pertussis toxin was also reported in S. paratyphi A (McClelland et al., 2004). The product of the ORF designated artA is similar to putative pertussis-like toxin subunit in S. typhi (STY1890) and S. paratyphi A (SPA1609), and to the S1 subunit of pertussis toxin, which is the catalytic subunit, an ADP-ribosyltransferase toxin. The product of artB is most similar to a hypothetical periplasmic protein in S. typhi (STY1364) and S. paratyphi A (SPA1188). ArtB is also similar to the putative pertussis-like toxin subunits of S. typhi (STY1891) and S. paratyphi A (SPA1610), which have a low but
convincing similarity to the S1 and S3 binding subunits of pertussis toxin. Pertussis toxin interferes with signal transduction by ADP-ribosylation of the G~i~ regulatory component of adenylate cyclase, and it is this action that is believed to be responsible for various physiological and cellular effects of the toxin (Bokoch et al., 1983; Katada & Ui, 1982). ADP-ribosyltransferase toxins are broadly distributed among highly pathogenic bacteria and are the primary cause of various severe human diseases such as diphtheria, cholera and pertussis. All of these toxins belong to the AB subunit class, where the A subunit is the toxic moiety that carries the active site and the B subunit is required for receptor binding and for translocation of fragment A across the host’s cell membrane (Burnette, 1994; Merritt & Hol, 1995). Therefore ArtA and ArtB may be the A and B subunits, respectively, of a novel AB toxin. Since both artA and artB have signal peptides, these polypeptides may be exported across the cytoplasmic membrane.

artA and artB were identified in adjacent sequences of a 0·4 kb HindIII fragment. The sequence of this 0·4 kb fragment shows homology to a segment of temperate phage ST64B which was isolated from S. typhimurium DT64 (Mmolawa et al., 2003). This phage is induced by mitomycin C, but is not able to produce plaques on a wide range of isolates (Mmolawa et al., 2002). Furthermore, the flanking region of artAB was found to contain another prophage or putative prophage-like sequences. We examined the inducibility of temperate phage using mitomycin C; however, we were not able to detect plaques of phage without ST104 with indicator strain LT2 by the analysis of plaque hybridization (data not shown). Presence of a stronger signal when the artA probe was hybridized with DNA obtained from a mitomycin C-induced S. typhimurium DT104 strain compared with a non-induced strain suggested that artA resides within a prophage other than ST104. Bacteriophages have played a critical role in the evolution of many bacterial pathogens (Wagner & Waldor, 2002). Toxins of a number of both Gram-positive and Gram-negative pathogens are encoded in the genomes of temperate bacteriophages. The structural genes encoding diphtheria toxin, cholera toxin and Shiga toxin 1 and 2 are all phage-encoded. The diverse bacteriophages that encode such virulence factors provide a means for the horizontal transmission of virulence genes within a bacterial population. We screened S. typhimurium strains for the presence of artA. Southern blotting analysis of the genomic DNA of S. typhimurium strains with a DIG-labelled probe for artA revealed that artA sequences are present in the genomes of all of the 12 DT104 isolates and four strains which are grouped into the same cluster as DT104 by PFGE and AFLP analysis, irrespective of their origin. The artA sequence was detected in the non-DT104 strain NCTC 73, which was isolated from humans in France in 1917; however, the other 13 S. typhimurium strains were all assumed to lack the artA locus. The results presented here suggest that phage-mediated recombination has played a critical role in acquisition of art genes in S. typhimurium DT104. Since no hybridization signal with an artA probe was observed in the phage particle fraction of strain NCTC 73 (data not shown), artA may not be encoded by the prophage in this strain. The artAB genes in DT104 may have originated from S. typhi, S. paratyphi A or from a strain such as NCTC 73.

Surveillance programme data show that the incidence of salmonellosis in cattle in Japan, especially in adult cattle, has increased continuously since 1992. An increased frequency of isolation of DT104 from cattle has also been noted in Japan since 1992 (Sameshima et al., 2000; Tamada et al., 2001). Some questions remain about the nature of the possible increased virulence of drug-resistant DT104 strains, since infection by DT104 is more likely to lead to hospitalization for a human or death of cattle than infection by a non-DT104 S. typhimurium (Glynn et al., 1998). Thus, these case control studies suggest that DT104 may be hypervirulent compared to antibiotic-susceptible strains of S. typhimurium (Glynn et al., 1998). A study of virulence in S. typhimurium can be performed by assessing the cellular invasion capabilities of individual isolates using a tissue culture assay. However, the ability of DT104 isolates to survive within murine peritoneal macrophages and to

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**Fig. 6.** Ethidium-bromide-stained gel containing DNAs extracted from the phage particle fraction of strain U1 (lanes 1 and 2), and their Southern hybridization results with an artA probe (lanes 3 and 4). Samples in lanes 1 and 3 were obtained without mitomycin C induction, whereas those in lanes 2 and 4 were with induction. Sizes of DNA markers are indicated to the left (lane M).
invade cultured epithelial cells, and the level of their lethality in mice, have been assessed and failed to demonstrate that DT104 isolates are more virulent than non-DT104 isolates (Allen et al., 2001). Recently, it was reported that certain strains of DT104 secrete a putative cytotoxin, Clg, which is similar to a collagenase-like protein (Wu et al., 2002). Clg exerts cytopathic effects that mimic the DT104-mediated cytotoxicosis (Carlson et al., 2001; Wu et al., 2002). The clg gene is present in Salmonella and other Gram-negative pathogens even though its transcripts were not detected in vitro (Wu et al., 2002). Furthermore, the addition of ADP-ribosyltransferase toxin could lead to an apparent enhanced virulence phenotype. In order to answer the questions surrounding the pathogenicity of DT104, further studies are now in progress to determine whether the ADP-ribosyltransferase toxin gene homologue, which is common among DT104 isolates, is expressed, and is active and exported.

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